The Effects of VEGFA/VEGFR interference on cell survival and angiogenesis in AML

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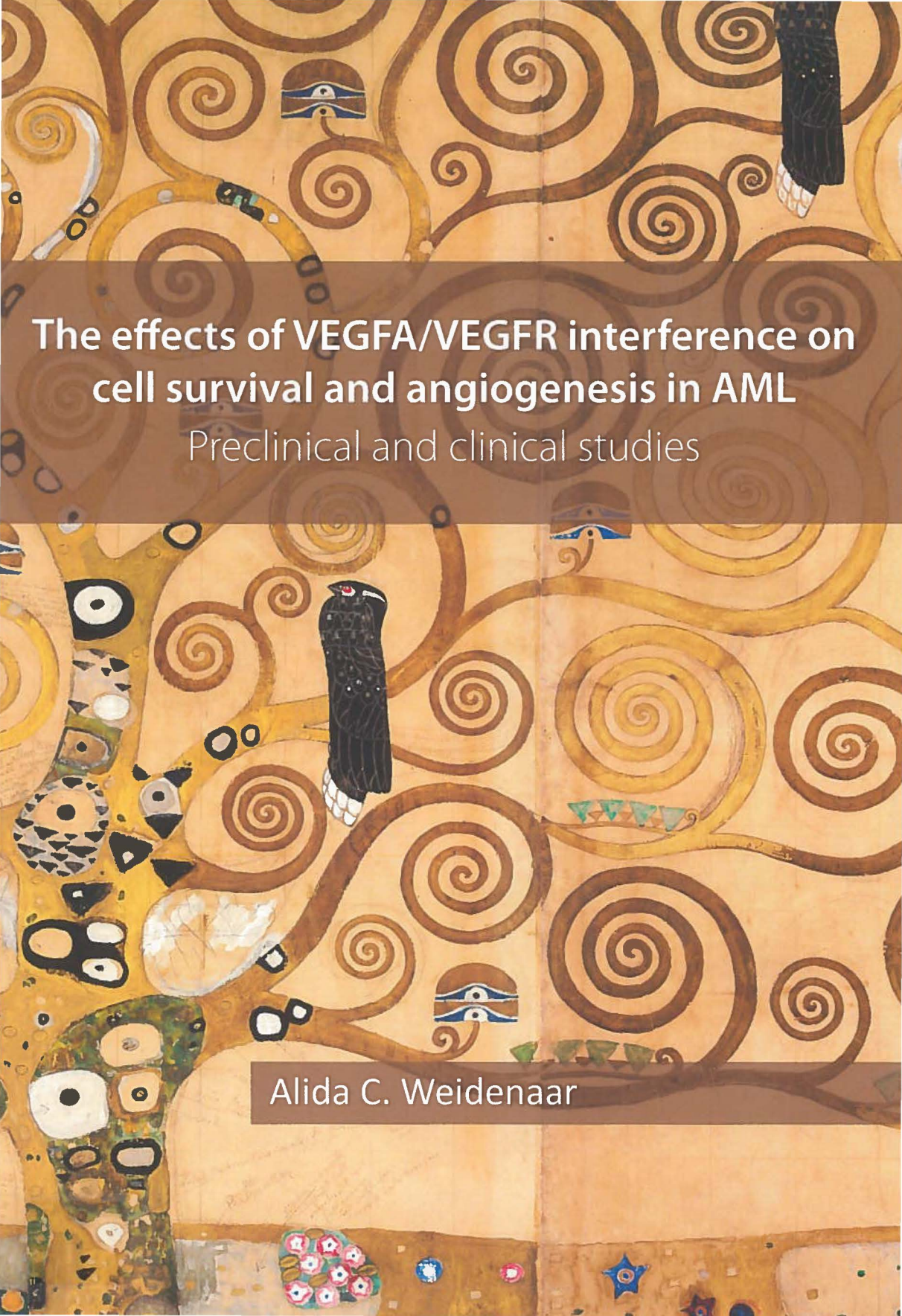

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Preclinical and clinical studies

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Stellingen

behorende bij het proefschrift

The effects of VEGFA/VEGFR interference on cell survival and angiogenesis in AML

Preclinical and clinical studies

1. De remming van verschillende receptor tyrosine kinases door PTK787/ZK 222584 resulteert in een afname van intracellulaire signaaltransductieroutes, zorgt voor een verminderde uitgroei van Leukemie Initiërende Cellen, en is daardoor een (mogelijk) waardevolle therapie. (dit proefschrift)

2. Hoewel VEGFA een onafhankelijke prognostische factor is voor AML overleving, blijkt het gebruik van een VEGFA-antilichaam niet afdoende. (dit proefschrift)

3. De interactie met de micro-omgeving maakt dat VEGFA additionele tumorgroei induceert, waarbij een rol voor TGF-ß signaaltransductie lijkt weggelegd. (dit proefschrift)

4. De vaatmorfologie in het beenmerg van AML patiënten laat drie basispatronen zien die gerelateerd zijn aan de behandeluitkomst. (dit proefschrift)

5. The obvious thing is to think about what we could remove from our diet. But a completely opposite approach is: can we eat to starve cancer? Food is a medicine that we take three times a day. *William Li*

6. Als een AIOS een deel van de opleiding zelf moet financieren, komt het salaris rond het minimumloon te liggen; een absurde situatie.

7. In de huidige tijd is het nog steeds zo dat vrouwen met hoge hakken en korte rokjes letterlijk en figuurlijk hogerop komen.

8. Binnen de perken zijn de mogelijkheden even onbeperkt als daarbuiten. *Jules Deelder*

9. Insanity is doing the same thing over and over again and expecting different results. *Albert Einstein*

10. Het is dat PEP-kuur een afkorting is (van Post Expositie Profylaxe), anders zou het deze opbeurende naam niet mogen dragen.

11. Als alles lukt wat je probeert, probeer je niet genoeg.

Alida C. Weidenaar, 2012

cm6.

rijksuniversiteit groningen

The effects of VEGFA/VEGFR interference on cell survival and angiogenesis in AML

Preclinical and clinical studies

Proefschrift

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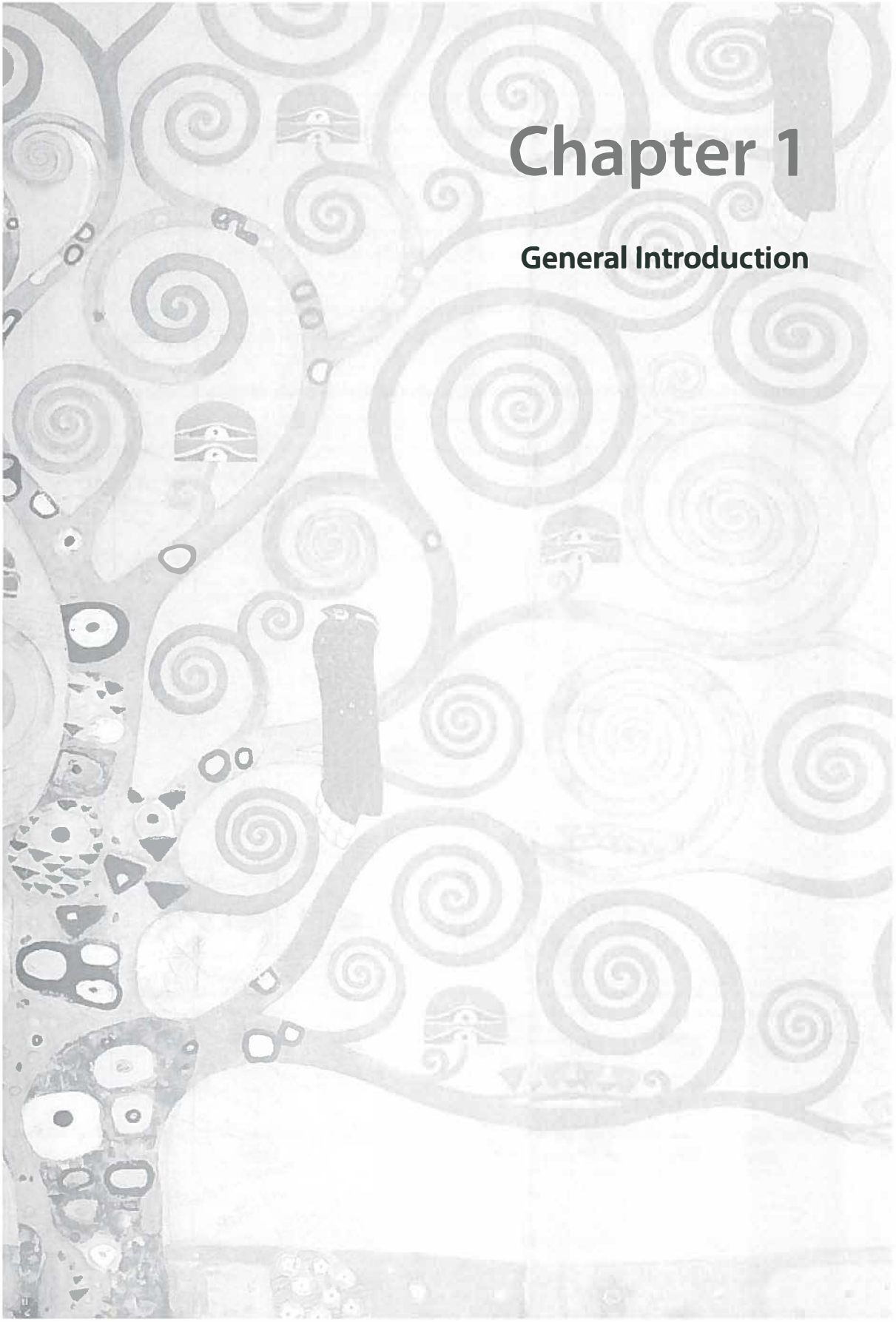
	Prof. dr. E. Vellenga
	Prof. dr. B. de Moerloose

Your patient has no more right to all the truth you know
than he has to all the medicine in your saddle-bags. . .
He should get only just so much as is good for him.
Oliver Wendell Holmes, 1871

Paranimfen: Annet Aukes
Kim Kampen
Tiny Meeuwsen-de Boer

Contents



Chapter 1

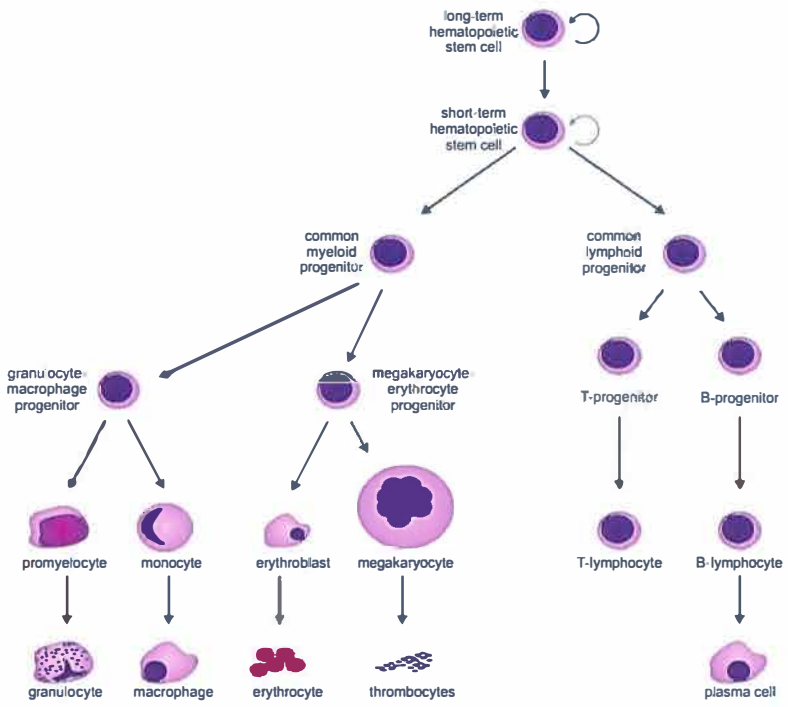
General Introduction

Normal and malignant hematopoiesis

Hematopoiesis is a hierarchically well-organized process by which all the different cell lineages that form the blood and immune system are generated from a common pluripotent stem cell, the Hematopoietic Stem Cell (HSC). These stem cells mainly reside in a quiescent state in the bone marrow and are defined by the ability to self-renew and undergo differentiation. Self-renewal is the process whereby, upon cell division, the mother cell gives rise to two daughter cells of which at least one daughter cell retains the characteristic of the parental cell. In contrast, progenitor cells differentiate into lineage-specific progenitor cells, generating different mature blood cells (Figure 1) [1]. Normally, only mature blood cells enter the blood stream.

Malignant hematopoiesis might occur when the control over normal proliferation and /or differentiation is lost, resulting in the development of a hematological malignancy, e.g. leukemia. Leukemia is characterized by a clonal expansion of immature blasts in the bone marrow and can develop at any stage in one of the hematopoietic cell lineages [2]. A differentiation block in the myeloid (earlier also called nonlymphoid) lineage results in 'myeloid leukemia', whereas interruption of differentiation in the lymphoid lineage induces 'lymphoblastic leukemia'. The disease can further be subdivided into 'acute leukemia' or 'chronic leukemia', e.g. based on clinical presentation. This thesis focuses on Acute Myeloid Leukemia (AML).

Figure 1. Hematopoiesis.

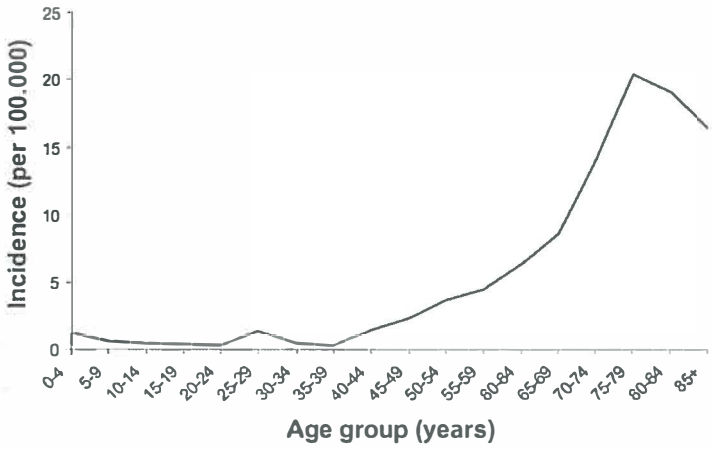


Acute Myeloid Leukemia

Epidemiology and Prognosis

AML occurs in children as well as in adults [3]. In the Netherlands, about 20 children per year are diagnosed with AML, and the incidence increases with age (Figure 2). Chemotherapeutic drugs are considered standard of care for pediatric and adult patients with AML, including stem cell transplantation for a selected group of patients. Despite an initial Complete Remission (CR) rate of 90% in pediatric AML patients, 30-40 % of these patients relapse [4-6]. The 5-year overall survival rate of AML is approximately 60% in pediatric AML patients. In adult AML patients a CR rate of 40-75% is seen, with increasing age as an important negative prognostic factor. Long-term survival (5 years) of the adult patients is ranging from approximately 40% in adults aged 18-60 years to 10% in the older patients [3,7-12].

Figure 2. Incidence rate of AML in the Netherlands.



Source: Dutch Cancer Registry (NKR) 2008.

Clinical presentation

Acute myeloid Leukemia is characterized by an increased number of immature myeloid cells in the bone marrow [7]. The clinical presentation of patients with AML is usually caused by leukemic infiltration of the normal bone marrow, which result in hematopoietic insufficiency e.g. thrombocytopenia, granulocytopenia, anemia and leukocytosis. The symptoms are mainly nonspecific and include fatigue, infections, dyspnea, and hemorrhage.

Classification

AML is a heterogeneous disease and classification of different AML subtypes is based on morphologic, cytochemical, and immunophenotypic features of the neoplastic cells to establish their lineage and

degree of maturation. The first most comprehensive morphologic-histochemical classification system was developed by the FAB (French-American-British) Group [13-15]. This classification system categorizes AML into subtypes primarily based on morphology and immunohistochemical detection of lineage markers. Recently, the World Health Organization (WHO) published a new classification system for hematopoietic neoplasms that incorporate those disease characteristics that have been proved to have clinical and biologic relevance into a useful, working nomenclature [16-18]. The WHO classification is listed in Table 1.

Table 1. WHO classification of AML (World Health Organization 2008)

Acute myeloid leukemia and related neoplasms
1. Acute myeloid leukemia with recurrent genetic abnormalities
AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
APL with t(15;17)(q22;q12); *PML-RARA*
AML with t(9;11)(p22;q23); *MLLT3-MLL*
AML with t(6;9)(p23;q34); *DEK-NUP214*
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*
AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM15-MKL1*
Provisional entity: AML with mutated NPM1
Provisional entity: AML with mutated CEBPA
2. Acute myeloid leukemia with myelodysplasia-related changes
3. Therapy-related myeloid neoplasms
4. Acute myeloid leukemia, not otherwise specified
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Acute erythroid leukemia
Pure erythroid leukemia
Erythroleukemia, erythroid/myeloid
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
5. Myeloid sarcoma
6. Myeloid proliferations related to Down syndrome
Transient abnormal myelopoiesis
Myeloid leukemia associated with Down syndrome
7. Blastic plasmacytoid dendritic cell neoplasm

Leukemic Stem Cell/ Leukemia Initiating Cell

Leukemia is thought to arise from a few Leukemia Initiating Cells (LICs), also known as Leukemic Stem Cells (LSCs). Investigations into the origin of LICs have revealed that these cells are strikingly similar to normal HSCs, with respect to their ability for self-renewal and cell-surface markers [19-23]. Given these shared attributes, it has been proposed that LICs may be derived from normal HSCs that have acquired an enhanced self-renewal program as a result of transforming mutations.

The first characterization of the LIC was described by Dick et al. in 1994, when they discovered that the CD34+CD38- cell fraction was enriched for LICs since these cells were capable of initiating leukemia in NOD/SCID mice [19]. In that time it was believed that only CD34+CD38- cells can transfer leukemia to NOD/SCID mice, whereas cells with a CD34-CD38- phenotype cannot initiate the disease. More recent, it was reported that the LICs may also reside in the CD34+CD38+ population [24]. Other studies suggested that even a more mature cell type may be the leukemia-initiating cell. Taussig et al. published that leukemia-initiating cells from some nucleophosmin (NPM)-mutated AMLs, associated with low frequencies of CD34+ cells, reside within the CD34- fraction [25]. Moreover, the oncogene PML-RARα, a result of the AML M3 translocation t(15;17), was absent in the CD34+CD38- cells, whereas the CD34+CD38+ population contained this translocation [26]. Recently, it was shown that introduction of the PML-RARα oncogene in committed myeloid progenitors initiates leukemia by conferring self-renewal capacity [27]. Another example that a myeloid leukemic progenitor may be the origin of leukemia is the mixed lineage leukemia (MLL) fusion proteins which is produced by translocations involving the MLL gene on chromosome 11q23; it was shown that this oncogene conferred stem cell-like properties when transduced in committed hematopoietic progenitors [28,29].

Concluding, these data indicate that the phenotype of the leukemia-initiating cell is more heterogeneous than previously thought. The exact phenotype is still under debate. There is great interest in elucidating the exact phenotype of the LICs, particularly since it is thought that remaining LICs are responsible for or contribute to AML relapse.

Angiogenesis

The development of human cancers depends on the de novo formation of blood vessels, a process called angiogenesis. In physiological conditions, blood vessels are distributed at regular and closely spaced intervals and are organized into a well-organized hierarchy. Blood vessels are composed of endothelial cells that form the inner lining of the vessel wall, a basement membrane underlying these endothelial cells, and perivascular cells (referred to as pericytes, vascular smooth muscle cells or mural cells) that surround the basement membrane. In contrast, pathological angiogenesis consists of non-uniformly distributed, irregularly branched vessels and are not formed in a clear hierarchical pattern [30-32]. At diagnosis of AML an enhanced vessel density is found in the bone marrow compared with remission status bone marrow samples or normal controls, whereas, to our knowledge, morphology has not been studied yet [33,34].

Vascular Endothelial Growth Factor related to AML

The best-characterized pro-angiogenic factor is vascular endothelial growth factor A (VEGFA, also known as vascular permeability factor). VEGFA plays an essential role in vessel formation, since mice embryos lacking a single VEGF allele developed abnormal blood vessels and lethality [35]. VEGFA is known to be upregulated in many human tumors, and is associated with increased angiogenesis [36]. Transcription of VEGFA mRNA can be upregulated by a variety of effectors, including growth factors, cytokines and hypoxia [37]. Exposure to low oxygen concentrations occurs in tumor cells and increases VEGFA gene expression, with hypoxia-inducible factor-1 as the key mediator [38,39].

Co-expression of VEGFA and its tyrosine kinase receptors VEGFR1 (fms-related tyrosine kinase 1, FLT1) and VEGFR2 (kinase insert domain receptor, KDR) has been reported previously in AML [40]. VEGFA can exert its effect via binding to the two tyrosine kinase receptors, resulting in the formation of an autocrine loop. Binding of VEGFA to VEGFR2 induces dimerization and consequent phosphorylation of a subset of intracellular tyrosine residues [41]. Within the cell, VEGFR2 phosphorylation results in activation of different signaling pathways, including phosphatidylinositol-3-kinase (PI3K)/Akt signaling and mitogen activated protein kinase (MAPK)/ERK activation [42,43]. These signaling cascades promote cell survival by inhibiting apoptosis.

In (pediatric) AML, the levels of VEGFA protein in the cell, plasma and supernatant are an independent prognostic factor for disease-free and overall survival, CR and overall survival, and relapse-free survival respectively [44-46].

Scope of the thesis

The research described in this thesis aimed to explore the various mechanisms by which VEGFA promotes tumor progression in AML via autocrine and/or paracrine mechanisms, e.g. angiogenesis. Special attention was focused on new potential small molecule inhibitors and antibodies interfering with VEGF/VEGFR signaling, and its results on the autocrine (AML blast survival) and paracrine effects in AML.

In order to provide background on the role of VEGFA in hematological malignancies, we first discussed the vasculature in normal and pathological situations, and described the role of VEGFA as a key mediator in the malignant progression of multiple hematological malignancies. Furthermore, we outline the results of therapeutic interventions to interrupt the VEGF signaling pathway (**chapter 2**).

Chemotherapy still is the main treatment for AML, despite the fact that many cancer patients experience side effects when treated with chemotherapeutic drugs and 40% or more of the pediatric AML patients experience a relapse. In this study we hypothesized that inhibition of autocrine VEGFA effects result in decreased AML blast survival. Using total cell kill assays, we investigated the effect of the potentially less toxic drug PTK787/ZK 222584 upon leukemic cell death in AML cell lines and pediatric AML samples as well as in combination with a chemotherapeutic drug Amsacrine (**chapter 3**). PTK787/ZK 222584 (PTK/ZK, Vatalanib) is a small molecule tyrosine kinase inhibitor that is most potent against VEGFR2, but has

also effect on other tyrosine kinase receptors including VEGFR1, VEGFR3 (fms-related tyrosine kinase 4, FLT4), c-Kit (KIT also known as CD1 17 or SCFR), Platelet Derived Growth Factor Receptor beta (PDGFRβ) and colony-stimulating factor-1 receptor (CSFR1, also known as c-FMS).

AML is thought to be a stem cell disease and inefficient targeting of the LIC is considered responsible for relapse after achievement of a complete hematological remission. Since treatment with PTK787/ ZK 222584 induced leukemic cell death in AML cell lines and pediatric AML samples (chapter 3), we described in **chapter 4** the effect of VEGF/VEGFR-interference in LIC enhanced CD34+ pediatric AML cells. The microenvironment in which the primary AML cells reside (the stem cell niche) was mimicked by culturing CD34+ pediatric AML cells on MS5 stromal cells. Various methods of interference with VEGFA/VEGFR signaling were used.

Besides autocrine effects, AML derived and secreted VEGFA stimulates the microenvironment such as endothelial and stromal cells. The effects in the microenvironment augment and/or drive leukemic cell proliferation via paracrine effects. In **chapter 5**, we investigated the interaction between tumor microenvironment and HL-60 AML cells in response to tumor-derived VEGFA by s.c. inoculating tumor cells transduced with VEGFA or an empty vector in NOD/SCID mice. Gene expression profiling combined with mouse-specific cytokine arrays on the tumors were used to get more insight into the mechanism of VEGFA-promoted tumor growth.

In AML, an increase in bone marrow vessel count at diagnosis is seen, restoring to normal when a complete remission has been achieved. In the process of angiogenesis, VEGFA plays a key role. In **chapter 6**, we investigated the morphological appearance of the vasculature in AML bone marrow biopsies at diagnosis, quantified by stainings for endothelial cell markers. Immaturity status was determined and relations with VEGFA were analyzed.
Next, we used a larger cohort to validate the three defined vasculature patterns defined at diagnosis of AML (as described in chapter 6). In **chapter 7** we hypothesized that a specific vasculature pattern is related to clinical outcome and/or the sensitivity for antiangiogenic strategies such as Bevacizumab. For this study we were able to use a larger series of bone marrow biopsies from AML patients (aged >60 years) included in a randomized clinical trial of Bevacizumab in addition to standard chemotherapy (n=93).

Finally, **chapter 8** summarized the abovementioned results and placed these in a broader perspective.

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Chapter 2

Angiogenesis in hematological malignancies

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Introduction

For more than a decade the role of angiogenesis and vascular endothelial growth factor (VEGF) in relation to tumor growth has been the object of intense research. In 1939 and onwards, it was postulated that "the rapid growth of tumor transplants is dependent upon the development of a rich vascular supply" [1,2]. Soluble, diffusible factors were held responsible for this new vessel formation [3,4]. In 1971 Folkman proposed that anti-angiogenesis might be an effective strategy to treat human cancers [5]. The identification of VEGF as a potent, diffusible factor affecting vascular endothelial cells led to ongoing investigations focused on VEGF (also referred to as VEGFA) as a key molecule in physiological and pathological vessel formation [6,7]. Despite the knowledge that most of the steps in tumor growth are highly complex and multifactorial processes, VEGFA has been shown to be a prerequisite in tumor growth.

Angiogenesis in normal vasculature

Blood vessels are critical for maintaining cellular homeostasis in the human body and therefore all cells must reside within 100 µm to 200 µm of a capillary [8,9]. Angiogenesis is defined as blood vessel generation from preexisting blood vessels and already exists in embryogenesis [10]. After birth, angiogenesis still contributes to organ growth but during adulthood most blood vessels remain quiescent and angiogenesis occurs only in the cycling ovary and in the placenta during pregnancy, in response to exercise training, in wound healing and in inflammatory processes [11-13]. Angiogenesis in the adult human body is thought to be the result of a delicate balance between endogenous stimulators and inhibitors (Table 1). Angiogenesis is stimulated by growth hormones such as VEGFA, fibroblast growth factor (FGF), platelet derived growth factor (PDGF) and during hypoxic conditions. Endogenous inhibitors of angiogenesis include various anti-angiogenic peptides, hormone metabolites and apoptosis modulators (extensively reviewed [14,15]). In addition, vascular basement membrane components can modulate endothelial cell behavior in order to provide structural and functional support [16].

Angiogenesis is a multistep process and several cell types such as endothelial cells and inflammatory cells produce and release angiogenic factors (e.g. VEGFA) upon certain stimuli, for instance hypoxia. High levels of VEGFA are able to initiate vasodilatation and an increased vascular permeability of pre-existing capillaries and post-capillary venules [17-19]. This allows extravasation of plasma proteins, which lay down a provisional matrix to which activated endothelial cells migrate. Angiopoietin (ANGPT)-2 binding to the Tie-2 receptor tyrosine kinase (TEK) is thought to result in loosening of pericyte coverage [20]. The activated endothelial cells start to release matrix metalloproteases (MMPs) and serine proteases which in turn degrade the basement membrane. After degradation of the basal membrane endothelial cells will be able to migrate from the original vessel walls towards the angiogenic stimuli originating from the injured tissue. Following this migration endothelial cells start to proliferate into the surrounding matrix and form solid sprouts connecting neighboring vessels. A vascular basal lamina is produced around the newly formed blood vessels upon disappearance of ANGPT2 and upregulation of ANGPT1, which

attracts and stabilizes pericytes and smooth muscle cells, finally resulting in fully mature blood vessels (nicely reviewed[21]).

Normal blood vessels are distributed at regular and closely spaced intervals and are organized into a hierarchy of elastic and muscular arteries, arterioles, capillaries, postcapillary venules and small and large veins (Figure. 1).

Table 1: Pro- and anti-angiogenic factors

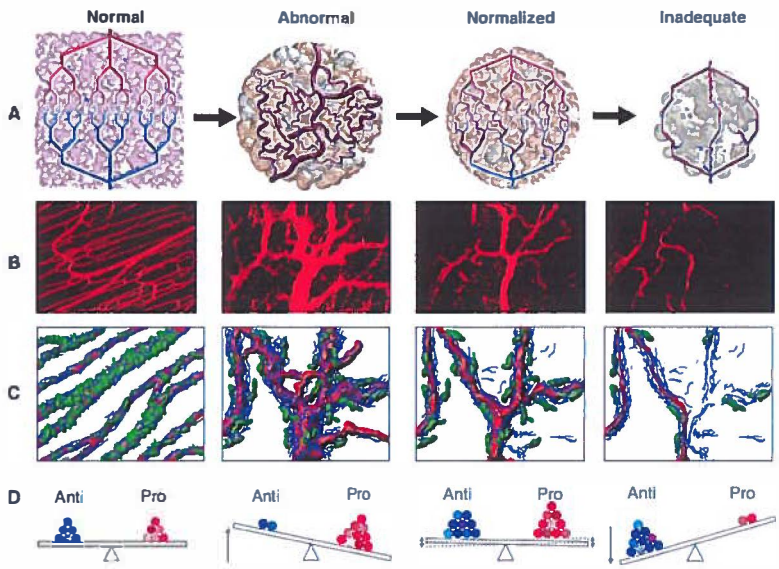
Pro-angiogenic factors	Anti-angiogenic factors
Acidic and basic fibroblast growth factor (FGF)	Angiopoetin-2
Agiogenin	Angiostatin
Angiopoitin-1	Arrestatin
Epidermal growth factor (EGF)	Canstatin
Folistatin	Endostatin
Granulocyte colony-stimulating factor (G-CSF)	Fibronectin
Granulocyte-macrophage colony stimulating factor (GM-CSF)	Interferon-α,β,γ
Hepatocyte growth factor (HGF)	Interleukin-1
Insulin-like growth factor (IGF)	Interleukin-12
Interleukin-2	Interleukin-18
Interleukin-6	Maspin
Interleukin-8	Platelet factor 4
Leptin	Restin
Matrix metalloproteinases (MMP)	Retinoic acid
Placenta growth factor	Soluble VEGF receptor
Platelet activating factor	Thrombospondin
Platelet-derived derived growth factor (PDGF)	Tissue inhibitors of metalloproteinases (TIMP)
Platelet-derived epidermal growth factor	Tumstatin
Pleiotrophin	Vasostatin
Proliferin	
Prostagladins E1, E2	
Transforming growth factor (TGF-α,β)	
Tumor necrosis factor-α	
Vascular endothelial growth factor (VEGF)	
Vascular integrin αvβ3 (vitaxin)	

Angiogenesis in pathological vasculature

In contrast to normal physiological angiogenesis, where new vessels rapidly mature and become stable, new blood vessels in pathological angiogenesis are non-uniformly distributed, irregularly branched and

are not formed in a clear hierarchical pattern (Figure. 1). Animal models showed that tumor vessels are dilated, immature (i.e. pericyte coverage is missing or detached, basement membrane is missing or too thick) and the vascular density is heterogeneous (Figure. 1C) [22-24]. An important feature of tumor blood vessels is that they fail to become quiescent, enabling the constant growth of new tumor vessels. The vascular network in tumors is often leaky and hemorrhagic, leading to an elevated interstitial pressure in the tumor resulting in hypoxia and acidosis [25]. These vessel characteristics make the delivery of therapeutics to solid tumors highly inadequate [26]. Rakesh Jain postulated the hypothesis that correcting the structure and function of tumor vessels could normalize the tumor microenvironment and eventually improve the treatment by more efficient delivery of drugs and oxygen to the targeted cancer cells [27]. Vessel normalization may be achieved by correcting the imbalance of angiogenic and anti-angiogenic factors in favor of anti-angiogenic factors. The high vascular permeability may be accomplished by the high levels of VEGFA which result in induction of Src regulated pathways [28-30]. Therefore, blocking the VEGF/VEGFR signaling might normalize tumor vasculature.

Figure 1. Proposed role of vessel normalization in the response of tumors to antiangiogenic therapy.



(**A**) Tumor vasculature is structurally and functionally abnormal. It is proposed that antiangiogenic therapies initially improve both the structure and the function of tumor vessels. However, sustained or aggressive antiangiogenic regimens may eventually prune away these vessels, resulting in a vasculature that is both resistant to further treatment and inadequate for the delivery of drugs or oxygen. (**B**) Dynamics of vascular normalization induced by VEGFR2 blockade. On the left is a two-photon image showing normal blood vessels in skeletal muscle; subsequent images show human colon carcinoma vasculature in mice at day 0, day 3, and day 5 after administration of VEGR2-specific antibody. (**C**) Diagram depicting the concomitant changes in pericyte (red) and basement membrane (blue) coverage during vascular normalization. (**D**) These phenotypic changes in the vasculature may reflect changes in the balance of pro- and antiangiogenic factors in the tissue [reprinted, with permission from the American Association for the Advancement of Science [168-170]].

Angiogenic switch

Spontaneously clustering tumor cells are usually not angiogenic at first [31]. For cancer progression a phenotypic switch to angiogenesis is required, a so-called angiogenic switch. This switch is more than an increase of pro-angiogenic stimuli; it is thought to be the net result of positive and negative regulators, a crosstalk between these factors and their receptors, as well as interaction with vasculature supporting cells, such as endothelial progenitor cells, mesenchymal (stem) cells and niche maker cells. It is hypothesized that genetic control of the physiological levels of endogenous angiogenesis inhibitors is a line of defense against the conversion of dormant tumor cells to a malignant phenotype. The underlying phenomenon resulting in this phenotypic switch is still not clear, however ongoing genetic instability is suggested to have a role [32,33].

The key player VEGF

The vascular endothelial growth factor (VEGF) family includes VEGFA, placental growth factor (PLGF), VEGFB, VEGFC, VEGFD, VEGFE and VEGFF [34-38]. These proteins can bind to and exert their effect on two cell surface receptor families: the tyrosine kinase receptors (VEGFR) and the neuropilin (NRP) receptors. Three VEGF receptors have been identified, namely VEGF receptor 1 (FLT1), VEGF receptor 2 (KDR) and VEGF receptor 3 (FLT4) [39-41]. Until now two neuropilins (NRP1 and NRP2) have been described [42,43]. Members of the VEGF family exert their effects by binding to the transmembrane receptors, resulting in the formation of dimers in the plasma membrane. Interaction between dimers is thought to stimulate autophosphorylation of the receptor. In response to phosphorylation of the receptors intracellular signals are transmitted via signaling pathways such as mitogen activated protein kinase (MAPK) or the phosphatidylinositol 3-kinase (PI3-K/Akt) cascades [44,45]. These signals are essential to various cellular processes including control of cell growth, differentiation and migration [46].

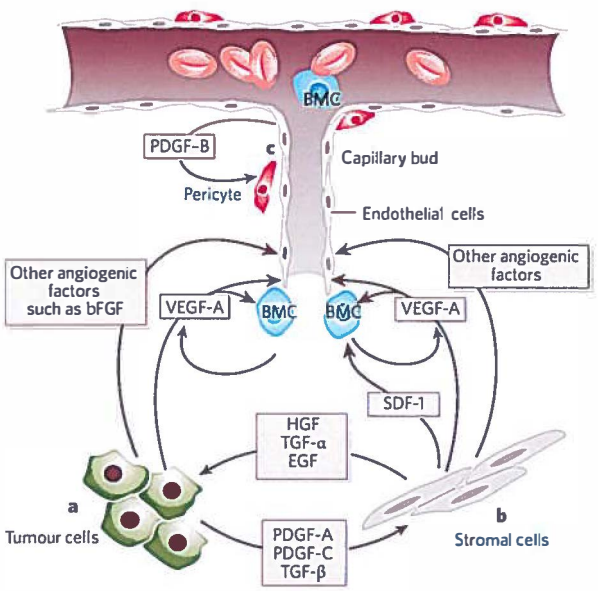
The last decade most of the research involving angiogenesis has been focused on VEGFA. In various malignancies VEGFA is associated with increased tumor growth (e.g. melanoma, lung cancer, breast cancer, colon cancer, rhabdomyosarcoma). Moreover, VEGFA is found to be an independent prognostic factor for outcome (e.g. breast, lung, colon/rectum, liver, gallbladder, bladder cancer and hematological malignancies such as Acute Myeloid Leukemia (AML)) [47-54]. More recently, several studies showed that high VEGFA expression resulted in tumor growth by autocrine and/or paracrine ways of action. Two different *paracrine* ways can be described: to induce angiogenesis (chemotactic signals from tumor cells might recruit stromal cells, which produce VEGFA leading to the process of angiogenesis) and to induce tumor cell proliferation (tumor cell derived VEGFA results in growth factor production in stromal cells and/or endothelial cells, which results in tumor cell proliferation; for instance described in AML [55]).

Bone marrow derived tumor growth supporting cells and circulating endothelial cells

Vasculature of tumors is predominantly formed by angiogenesis, but can also be developed by postnatal vasculogenesis: the formation of new blood vessels from Endothelial Progenitor Cells (EPCs)

which differentiate into mature Endothelial Cells [56]. These EPCs are defined as CD34+/CD133+/CD177+/KDR+ cells that originate in the bone marrow. EPC recruitment and mobilization have been positively correlated with an increase in VEGFA levels; in response to increased VEGFA levels at the site of the tumor, an increase in circulating EPCs was seen (Figure. 2) [57-59]. Moreover, CD133+ cells differentiate into mature-type adherent ECs and abolish the CD133 expression in response to VEGFA [60]. While it has been shown that these bone marrow derived EPCs contribute to the tumor vessel formation by incorporating into the endothelium [61], a second group of pro-angiogenic cells was described; this population, rather than the EPCs, was thought to home specifically to the tumor and was characterized by the expression of CD45+, TEK+, CD31- and CD11b+ [62]. Inhibition of those cells resulted in a significant reduction of tumor angiogenesis and growth. Finally, the existence of a third pro-angiogenic population was suggested, which is characterized by CD34, CXCR4 and FLT-1 expression and is implicated in the initiation and formation of metastases [63].

Figure 2. A few of the molecular and cellular players in the tumour/microvascular microenvironment.



(**A**) Tumour cells produce VEGF-A and other angiogenic factors such as bFGF, angiopoietins, interleukin-8, PlGF and VEGF-C. These stimulate resident endothelial cells to proliferate and migrate. (**B**) An additional source of angiogenic factors is the stroma. This is a heterogeneous compartment, comprising fibroblastic, inflammatory and immune cells. Recent studies indicate that tumour-associated fibroblasts produce chemokines such as SDF-1, which may recruit bone-marrow-derived angiogenic cells (BMC). The various hypotheses on the nature and role of such cells in angiogenesis and tumour progression are discussed in the text. VEGF-A or PlGF may also recruit BMC. Tumour cells may also release stromal cell-recruitment factors, such as PDGF-A, PDGF-C or transforming growth factor (TGF)-β. A well-established function of tumour-associated fibroblasts is the production of growth/survival factor for tumour cells such as EGFR ligands, hepatocyte growth factor and heregulin. (**C**) Endothelial cells produce PDGF-B, which promotes recruitment of pericytes in the microvasculature after activation of PDGFR-β. HGF, hepatocyte growth factor. [reprinted with permission from Macmillan Publishers Ltd [171]].

A related circulating cell population is the group of the circulating endothelial cells (CECs); these cells express CD146+, CD133-, vWF+, VE-cadherin+, CD45- and CD14-. The presence of CECs has been described as a useful marker for vascular damage; mature CECs are thought to have sloughed off the vessel wall indicating endothelial damage [64]. Another approach is monitoring CECs as a marker for anticancer treatment; elevated levels of CECs have been described in several malignancies, including Multiple Myeloma (MM) and lymphoma. In MM, an elevated number of CECs was described, correlating with serum markers of disease activity [65]. High CEC levels at diagnosis of lymphoma is changed to normal when they achieve a complete remission (CR) following chemotherapy [66].

Anti-angiogenic concepts

The importance of new vessel formation and/or VEGFA production for tumor growth resulted in efforts to inhibit angiogenesis, leading to the development of anti-angiogenic concepts. In the past a differentiation has been made upon the goal of the anti-angiogenic treatment; three classes are described: anti-endothelial cell strategy (e.g. endostatin, thrombospondin), anti-VEGFA therapy (Bevacizumab or VEGFR inhibitors, such as PTK787/ZK 222584, SU5416, SU6668, GW786034) and vascular targeting. The difference between anti-endothelial drugs (Rx) and anti-VEGFA Rx is the target of the strategy; the former is directed to endothelial cells whereas the latter exert its effect on endothelial cells as well as on tumor cells. The third class, vascular targeting, is still a promising goal; it initiates a specific response to the already established tumor vascular supply [67]. Until now, however, without the recognition of tumor specific vessel epitopes, this remains a future perspective. Moreover, vascular disrupting agents will result in rapid necrosis and a chance of fast regrowth in the viable outlayer of a tumor. Mice models and *in vitro* studies have demonstrated the feasibility of these anti-angiogenic concepts. However, anti-angiogenic compounds are often characterized by a short half-life, and the delivery at the tumor site remains difficult. Therefore, treatment results only in marginal changes of angiogenesis.

Changes of angiogenesis can also be achieved by chemotherapeutics drugs. Tumor-associated endothelial cells proliferate at a much lower rate than cancer cells and are therefore weakly inhibited by conventional chemotherapeutic protocols. During the normal rest periods of chemotherapy those endothelial cells may recover rapidly, resulting in regrowth of the tumor. Therefore, frequent administration of lower doses of cytotoxic agents, called 'metronomic scheduling', might be a better strategy to impede with repair of those endothelial cells. Preclinical models have shown that metronomic dosing induced repetitive peaks of endothelial cell apoptosis whereas the conventional scheduling showed a single wave of endothelial cell damage that was largely repaired at the end of the rest period [68,69]. Although the metronomic dosing resulted in a delay of tumor growth, these tumors will eventually escape control and relapse. As a result, metronomic schedules have been administered in combination with anti-angiogenic compounds which resulted in a significant increased therapeutic efficacy [70-72].

Another explanation for the repair of tumor vasculature during the prolonged drug-free periods between the cycles of high-dose chemotherapy is the mobilization and viability of bone-marrow derived EPCs as part of an adaptive response to the chemotherapy-induced myelosuppression [73]. A few

days after treatment with high dosages chemotherapy a high number of EPCs were mobilized, which mediates repair of the vasculature. In contrast, metronomic dosing schedules suppressed EPC numbers and induced the apoptosis of EPCs [74]. Because other studies showed the contribution of circulating endothelial cells to the tumor growth [75,76], the antivasculogenesis process of metronomic scheduling may also be mediated through reduction of EPC mobilization and viability.

VEGFA is known to promote the mobilization of bone marrow-derived EPCs, which may subsequently differentiate into CECs [77]. This mobilization is mediated by VEGFA binding to both FLT1 and KDR [78]. VEGFA is also thought to promote survival by activating antiapoptotic pathways in EPCs and CECs [79-81]. Therefore, treatment with anti-VEGF/VEGFR antibodies may also result in beneficial effects on tumor vasculature by a reduction of circulating endothelial cells mobilization as well as by an increase in apoptosis [82].

Initiation of the development of anti-VEGFA strategies, targeting both endothelial cells and tumor cells, followed the knowledge that VEGFA was an important player in tumor growth. Pharmaceutical compounds for humans interfering with VEGF/VEGFR signaling are currently under development. At this moment a humanized anti-VEGFA monoclonal antibody (Bevacuzimab; Avastin, Genentech, South San Francisco, CA) has been approved by the Food and Drug Administration as a first line treatment for metastatic colorectal cancers. Phase II/III studies in various malignancies are already reported to be promising for VEGF/VEGFR interfering drugs in addition to chemotherapeutic strategies; this accounts for anti-KDR antibodies, small molecules inhibiting KDR signal transduction and VEGFR chimeric proteins [83,84]. For an overview of the studies see Table 2.

Table 2: Clinical trials of anti-angiogenic drugs

Drugs	Trial	Mechanism
Drugs that block breakdown of extracellular matrix		
Dalteparin	Phase II ovarian cancer, advanced colon cancer	Inhibition of blood coagulation
Marimastat	Phase III, small cell lung, and breast cancer	Synthetic inhibitor of matrix metalloproteinases (MMPs)
COL-3	Phase I/II, brain	Synthetic MMP inhibitor Tetracycline derivative
Neovastat	Phase III, renal cell cancer, non-small cell lung cancer	Naturally occurring MMP inhibitor
BMS-275291	Phase II/III, advanced or metastatic non small cell lung cancer	Synthetic MMP inhibitor
Drugs that inhibit endothelial cells directly		
ABT-510	Phase I/Phase II, advanced head and neck cancer; Phase I, advanced solid tumors	Thrombospondin analogue
NGR-TNF	Phase I, advanced solid tumors	Tumor vasculature, TNF-α
Comretastatin A4 Phosphate	Phase I, advanced solid tumors; Phase II, advanced anaplastic thyroid cancer	Microtubule-disrupting agent
	Phase II, hormone-refractory metastatic prostate cancer	Induces the cytokines TNF-α, serotonin and nitric oxide

Table 2: Clinical trials of anti-angiogenic drugs *(Continued)*

Drugs	Trial	Mechanism
Drugs that inhibit endothelial cells directly		
Lenalidomide	Phase I-II, several cancer types; Phase III, multiple myeloma and refractory B-cell chronic lymphocytic leukemia; Phase IV, multiple myeloma	Derivative of thalidomide, immunomodulatory drug, exact mechanism not known
LY317615	Phase I-II, several cancer types including; Phase III glioblastoma and lymphoma	Protein Kinase C-ß–Selective Inhibitor
Soy Isoflavone	Phase II, bladder cancer, stage IV breast cancer, malignant melanoma, renal clear cell carcinoma and pancreatic cancer	Blocks the activity of Protein Tyrosine Kinase, topoisomerase II and MMP9 and downregulates the expression of 11 genes, including VEGF
Tamoxifen Citrate	Phase I-IV, primarily breast cancer	Anti-estrogen
Thalidomide	Phase I-III, several cancer types, primarily multiple myeloma	Unknown
Squalamine	Phase I, advanced cancers, Phase II, non small cell lung cancer, ovarian cancer	Extract from dogfish or shark liver; inhibits sodium, hydrogen exchanger NHE3
Endostatin	Phase I, solid tumors	Inhibition of endothelial growth
Drugs that block the activators of angiogenesis		
ADH-1	Phase I, incurable solid tumors	Blocks N-cadherin
AMG 706	Phase I-II, several cancer types, including breast cancer and non-small cell lung cancer	Multi-kinase inhibitor targeting VEGF, PDGF and c-KIT receptors
Bevacizumab/ Avastin	Phase I-IV, several cancer types; Phase IV, primarily colorectal cancer and non-small cell lung cancer	Antibody against VEGF
AZD2171	Phase I-III, several cancer types; Phase III, primarily colorectal cancer, breast cancer, and non-small cell lung cancer	VEGF Receptor-2 Tyrosine Kinase Inhibitor
Bay 43-9006	Phase I-III, several cancer types; Phase III, renal cell cancer, non-small cell lung cancer, and melanoma	VEGF Receptor-2 and 3, FLT3 and c-KIT Tyrosine Kinase inhibitor
BMS-582664	Phase I, solid tumors; Phase II, metastatic hepatocellular cancer, advanced gastrointestinal malignancies	VEGF Receptor-2 Tyrosine Kinase Inhibitor
CHIR-265	Phase I, advanced metastatic melanoma	VEGF Receptor-2 Tyrosine Kinase Inhibitor
GW786034	Phase I-III, several cancer types; Phase III, renal cell cancer	VEGF Receptor-2 Tyrosine Kinase Inhibitor
PI-88	Phase II, metastatic melanoma, prostate cancer	Inhibits heparinase activity and heparin sulfate binding to FGF and VEGF
PTK787/ZK 222584	Phase I-II, several cancer types; Phase II, glioblastoma, pancreatic cancer, myelodysplastic syndromes, multiple myeloma, breast cancer, gastrointestinal tumors, and meningioma	Multi-kinase inhibitor targeting VEGF, PDGF, and c-KIT receptors

Table 2: Clinical trials of anti-angiogenic drugs *(Continued)*

Drugs	Trial	Mechanism
Drugs that block the activators of angiogenesis		
Suramin	Phase I, bladder cancer; Phase I-II, breast cancer	Inhibits growth factors and receptors, including EGF, PDGF, FGF and VEGF
SU11248	Phase I-III, several cancer types; Phase III, gastrointestinal stromal tumors, renal cell carcinoma, breast cancer, pancreatic island tumors, and colorectal cancer	Multi-kinase inhibitor targeting VEGF, PDGF, FLT3 and c-KIT receptors
XL184	Phase I, advanced malignancies	Inhibits MET, VEGF receptor-2, FLT3, c-KIT, and TIE2
ZD6474	Phase I-III, several malignancies; Phase III, non-small cell lung cancer	Inhibits VEGF receptor, and EGF receptor activity
SU5416	Phase I-III solid tumors; Phase I, AML; Phase II, Multiple Myeloma	Selective inhibition of VEGF receptor activity
SU6668	Phase I, advanced solid tumors	Blocks VEGF, FGF, and PDGF receptor signaling
Interferon-α	Phase II-III advanced solid tumors	Inhibition of bFGF and VEGF production
Drugs that inhibit endothelial-specific integrin/survival signaling		
ATN-161	Phase I-II, recurrent intracranial malignant glioma	Integrin antagonist
EMD121974	Phase I, advanced solid tumors, lymphoma; Phase II, metastatic androgen-independent prostate cancer, glioblastoma multiforme	Small molecule blocker of alpha-v-integrins present on endothelial cell surface
Drugs with non-specific mechanism of action		
Celecoxib	Phase II-III, prostate cancer	Highly selective COX-2 inhibitor
CAI	Phase I, studies in combination against solid tumors; Phase II, ovarian cancer, metastatic renal cell cancer	Inhibitor of calcium influx
Interleukin-12	Phase I-II, Kaposi's sarcoma	Upregulation of interferon-gamma and IP-10
IM862	Phase I, recurrent ovarian cancer; Phase II, metastatic cancers of the colon and rectal; Phase III, Kaposi's sarcoma	Unknown mechanism

The role of VEGF and anti-VEGF therapy in hematological malignancies

Multiple Myeloma

The prognostic relevance of increased angiogenesis in the field of hematological malignancies was for the first time observed in multiple myeloma (MM) [85,86]. Increased VEGFA levels are present in the serum of MM patients with advanced disease stages and associated with increased angiogenesis [87-89]. Moreover, VEGFA is identified to play a key role in sustaining angiogenesis in MM [90,91]. A paracrine loop has been described: it was shown that VEGFA is expressed and secreted by MM cells and this MM

derived VEGFA stimulates IL-6 secretion in Bone Marrow Stem Cells (BMSCs). Stromal cell derived IL-6 in its turn enhances MM cell proliferation, migration, survival and VEGFA production, thereby augmenting MM cell growth, one of the first described paracrine VEGFA loops [92,93]. Another autocrine loop has been demonstrated via FLT1. Therefore, it has been suggested that direct and/or indirect targeting of VEGFA and its receptors may provide a potent therapeutic approach for the related angiogenesis as well as for paracrine and autocrine mediated tumor growth.

The knowledge about increased tumor growth augmented by angiogenesis resulted directly in new anti-angiogenic treatment strategies. Thalidomide was used as an anti-angiogenic drug in MM and resulted in enhanced survival. Nowadays, it is demonstrated that the function of thalidomide seems not restricted to anti-angiogenesis; surrounding bone marrow stromal cells as well as myeloma cells themselves seemed to be targets of thalidomide [94].

To counteract angiogenesis and/or VEGFA more specifically, a new generation of drugs was used. Several first-generation small molecule VEGF receptor inhibitors, such as the receptor tyrosine kinase inhibitor PTK787/ZK 222584 (a joint development project between Novartis Pharmaceuticals, Basel, Switzerland and Schering AG, Berlin, Germany) and the pan inhibitor of VEGF receptors GW654652 (GlaxoSmithKline) showed significant anti-MM effects *in vitro* [95,96]. Recently, the small-molecule tyrosine kinase inhibitor of FLT1, KDR and FLT4, Pazopanib (GW786034B; GlaxoSmithKline), showed for the first time *in vivo* tumor inhibition in a mouse model [97]. At the moment a multicenter phase I/II study with single agent Pazopanib is ongoing in patients with relapsed or refractory MM.

Hodgkin Lymphoma

Angiogenesis and angiogenic factors have also been studied in malignant lymphomas. Although not much is known about the role of angiogenesis in Hodgkin Lymphoma (HL) one study showed an inverse relationship between microvessel density (MVD) and the stage of disease in patients with HL. It was hypothesized that in HL the angiogenic phenotype might be an early event and the angiogenic capacity is tempered and unable to keep pace with the growth of the neoplastic cells [98]. The prognostic role of VEGFA has been observed in HL as well, showing that both high pre-therapy and post-therapy VEGFA levels were independently predictive for a poor overall survival [99]. Another independent prognostic factor for HL is the amount of mast cells: Hodgkin patients with many mast cells in their tumor had a shorter relapse-free survival [100]. Those mast cells express several factors such as VEGFA, that can affect angiogenesis both directly and indirectly [101]. It was therefore hypothesized that the increase in angiogenesis was due to an increase of VEGFA produced by mast cells. However, so far a high microvessel count could not be correlated to mast cell count [102]. Until now no pre-clinical or clinical trials with anti-VEGF drugs have been reported for the treatment of Hodgkin lymphomas.

Non-Hodgkin Lymphoma

The role of angiogenesis and angiogenic factors is has been studied more extensively in Non-Hodgkin lymphomas (NHL) compared to HL. In contrast to the observations in HL, the angiogenesis increases

with tumor progression [103]. It is known that the lymph node biopsies in patients with NHL show an increased MVD compared to benign lymphadenopathies [104]. VEGFA has also been implicated in the overall disease course of patients with NHL; VEGFA levels were elevated in patients with NHL compared to normal individuals, the event-free survival rate was significantly higher in patients with baseline VEGFA levels and the response to therapy was significantly more adequate in VEGFA-negative patients compared to VEGFA-positive patients [105-107]. Furthermore, the serum concentration of VEGFA and/or bFGF is an independent prognostic factor for outcome [108,109].

NHL cells secrete VEGFA and express FLT1 and KDR, suggesting the presence of autocrine and paracrine pathways [110]. Moreover, most of the malignant lymphomas express VEGFC, which can bind to FLT4 and is involved in lymph angiogenesis. The level of lymph vessel density was significantly correlated to the expression levels of VEGFA and VEGFC.

In vivo mono-experiments showed that treatment with an inhibitor targeting tumor FLT1 or host VEGFR2 reduced established diffuse large B-cell lymphoma (DLBCL) xenograft growth, whereas targeting tumor KDR and host VEGFR-1 had no effect. Decreased tumor volumes correlated with increased tumor apoptosis and reduced vascularization, respectively, suggesting the existence of autocrine FLT1 and paracrine KDR mediated pathways in lymph angiogenesis [111].

Preliminary results of a phase II clinical trial with Bevacizumab (a recombinant monoclonal antibody against VEGFA) showed a well-tolerated and prolonged stabilization of disease in patients with relapsed, aggressive NHL [112]. Another approach is a small-molecule inhibitor; Sorefenib is an inhibitor of several kinases including Raf, VEGFR and platelet derived growth factor. Currently a phase I study with Sorefenib is ongoing for NHL.

Acute Myeloid Leukemia

At diagnosis of Acute Myeloid Leukemia (AML) pronounced vessel density is found in the bone marrow compared to remission status bone marrow samples or normal controls [113-115], whereas in bone marrow specimens of refractory AML vessel density remained high. The impact on outcome was described more recently. A study using dynamic contrast-enhanced magnetic resonance imaging (dMRI) (lumbar spine) measuring blood flow and perfusion as marker for angiogenesis showed that high blood flow/perfusion was correlated to a poor outcome [116].

A correlation between increased vessel density and high VEGFA was described previously [117,118]. The importance of VEGFA in AML was described by various independent studies. These studies demonstrated that VEGFA was an independent prognostic factor for therapeutic outcome [119,120]. Biological insights of VEGFA in AML showed that VEGFA was not only a key player in angiogenesis resulting in indirect tumor growth, but that VEGFA was also able to induce and/or support leukemic cell growth in other ways. First, an additional paracrine route of tumor enhancement was described related to the interaction with bone marrow stromal cells [121,122]. Two pathways were recognized; leukemic cell derived VEGFA lead to GM-CSF production of stromal cells, and this GM-CSF supports for leukemic cell proliferation and/or survival [123]. In another paracrine route leukemic cell derived growth factor expression resulted in VEGFA production

of stromal cells/endothelial cells. When leukemic cells are sensitive for VEGFA stimulation this route will also enhance tumor growth [124]. A second way in which VEGFA results in more leukemic cell proliferation and/or survival is an autocrine route [125]. It was shown that AML cells can produce VEGFA and express its receptor, which makes AML cells sensitive for VEGFA dependent proliferation. The downstream effects of VEGFA are mainly executed by KDR binding, resulting in increased AML cell survival and proliferation (via MAPK and PI 3-K/AKT signaling) and protection against apoptosis (via bcl-2 and mcl-1) [126-129]. These biological insights resulted directly in new anti-angiogenic and anti-VEGF treatment studies.

Clinical trials are ongoing for AML. A phase I study of SU11248, a Receptor Tyrosine Kinase (RTK) inhibitor of c-Kit, FLT-3, KDR and PDGFR in the treatment of patients with refractory or resistant AML induced partial remission of short duration [130]. In a phase II trial SU5416, a RTK inhibitor of c-kit, FLT-3 and KDR showed modest clinical activity [131]. In this particular study patients with AML blasts expressing high levels of VEGFA had a significantly higher response rate than patients with low VEGFA expression.

In another phase I study no significant responses to treatment with PTK787/ZK 222584 in patients with primary refractory or relapsed AML was shown. PTK787/ZK 222584 was also tested in patients with secondary AML. Monotherapy resulted in a stabilization of the disease; when PTK787/ZK 222584 was combined with chemotherapy one third of the patients achieved CR [132]. Another clinical approach for the treatment of AML was chosen by using Bevacizumab, an anti-VEGFA monoclonal antibody, following chemotherapy [133]. In adults with relapsed and refractory AML that were resistant to traditional chemotherapy, a combination of cytotoxic chemotherapy followed by Bevacizumab resulted in a complete remission in one third of the patients and an increase in the median disease free survival [134].

Clinical experiences in phase I and II studies showed that single agents often are suboptimal in the induction of a clinical response (partial or complete remission) in AML. Various independent studies demonstrated the activation of one of the three important signaling transduction pathways; PI3K/AKT pathway, PKCalpha phosphorylation and RAS/Raf/MEK/ERK pathway, and its relation with outcome [135-138]. Recently, it was shown that simultaneous activation of more than one signal transduction pathways confers poor prognosis in AML [139]. These results will have broad implications for the field of drug development; by tradition new agents are evaluated individually. Consequently, cross-activation between these pathways may suggest that a drug can be ineffective whereas in cooperation with drugs targeting multiple signaling transduction pathways the result might be more effective. Until now the possible role of these kinds of strategies related to outcome still unclear.

Acute Lymphoblastic Leukemia

The importance of VEGFA and angiogenesis in Acute Lymphoblastic Leukemia (ALL) is controversial. In the marrow of children with ALL a significantly elevated microvessel density was found compared to healthy controls. Moreover, the MVD dropped towards normal in remission [140,141]. However, there was no difference in MVD at presentation or remission from patients with a poor prognosis [142].

VEGFA levels were higher in patients with recurrent disease compared to those with newly diagnosed ALL. Relapse-free survival and overall survival were shorter in ALL patients with high VEGFA levels [143].

Recently, Avramis showed that increased VEGFA serum concentrations during induction are correlated with events and poor survival of standard-risk ALL pediatric patients [144]. In contrast, Aguayo showed that levels of HGF, TNF-α, and bFGF, but not of VEGFA, were found to be elevated in blood samples of patients with acute lymphoblastic leukemia (ALL) [145]. In addition, higher levels of interleukin-1 receptor, interleukin-8, FLT1 and KDR, but not VEGFA, were predictive of poor survival in adult ALL patients [146]. Moreover, another study reported that the serum levels of VEGF at time of diagnosis were significantly lower than in the control group and at time of remission [147].

In conclusion, controversial data are published on the topic of ALL cells and angiogenic factors; a better understanding of the complex interaction is needed. Currently, it is not clear what the potential role of therapeutic (i.e. anti-VEGF) interventions in ALL will be.

Chronic Myeloid Leukemia

An elevated MVD of the bone marrow is also one of the characteristics for patients with Chronic Myeloid Leukemia (CML) [148]. It has been shown that the morphology of the vessels in the bone marrow of CML patients differed from controls in that the vessels were more tortuous and branched. The characteristics of the microvessels and the MVD also appeared to be predictors of patient survival and progression [149]. In addition, patients with newly diagnosed CML were found to have an increased VEGFA plasma concentration compared to healthy controls [150]. CML patients with a higher VEGFA level had a shorter overall survival [151]. A reduced overall survival was also correlated with an upregulation of KDR [152]. Those data support the prognostic value of VEGFA and MVD in CML patients. Moreover, the number of VEGFA-positive bone marrow cells correlated significantly with the MVD [153].

One of the drugs used for the treatment of CML is imatinib mesylate (STI571, also known as Glivec), a specific inhibitor of Bcr/Abl tyrosine kinase activity. The oncogene Bcr/Abl induces expression of VEGFA [154]. An *in vitro* study demonstrated that imatinib inhibited VEGFA gene transcription by targeting the Sp1 and Sp3 transcription factors [155]. Therefore, this drug might also be a potent inhibitor of VEGFA signaling. SU5416, an inhibitor of KDR and other tyrosine kinase receptors, was tested in a phase II clinical trial of patients with myeloproliferative disorders (MPD), including 4 patients with CML. Unfortunately, patients did not benefit from this study [156].

Chronic Lymphocytic Leukemia

Patients with Chronic Lymphocytic Leukemia (CLL) had a significantly higher MVD measured in bone marrow biopsies than controls. This increased MVD also correlated significantly with the clinical stage of the CLL patients [157,158]. Furthermore, other parameters were considered to be useful for prognostics; the plasma levels of VEGFA, bFGF and HGF were significantly increased in CLL [159] and these high serum levels of VEGFA correlated with a poor clinical outcome [160]. A significantly shorter overall survival was also correlated with elevated levels of KDR [161]. In addition, it is known that CLL cells produce VEGFA [162] and that the receptors for VEGFA (FLT1, KDR and FLT4) were expressed in the majority of CLL patients [163], suggesting an autocrine loop that stimulates CLL growth. VEGFA was able to decrease the apoptotic

potential of CLL B cells significantly [164]; interruption of these pathways might therefore contribute to increased leukemic cell death. An *in vitro* study showed that inhibition of VEGF receptor activation with either tyrosine kinase inhibitors or VEGF neutralizing antibodies inhibited VEGF receptor phosphorylation, decreased p-STAT-3 (serine 727), Mcl-1, and induced cell death in CLL cells [165]. Until now, no clinical trials have been published.

Clinical perspectives for the next five years

Although anti VEGF/VEGFR therapy in clinical trials seems promising, the net result of anti VEGF/VEGFR therapy in human clinical trials is an increased survival for only a few months. The discrepancy between marked anti-tumor activity of VEGF/VEGFR inhibitors in, for instance, mouse-models and less promising clinical results when used as a single agent has resulted in the use of combination strategies. For the future the ideal balance for dosing and administration of anti-angiogenic drugs in combination with chemotherapeutics will be one of the hallmarks of research in this field; combined treatment with anti-angiogenic agents and chemotherapy seems to be more effective. In addition, broad apoptotic drugs against endothelial cells as well as tumor cells appear to function better than the cell cycle dependent drugs used in clinics nowadays. Combinations of several tyrosine kinase inhibitors covering a broad spectrum of receptors, in order to avoid the occurrence of escape mechanisms, also show promising results, and are therefore another future perspective in the field of anti-angiogenic drugs.

There are still many challenges in the molecular understanding of tumor vasculature. It was hypothesized that normalization of tumor vessels could result in improvement of the treatment by a more efficient delivery of drugs to the targeted cancer cells. It still needs to be answered how these vessels will 'normalize'. Furthermore, the challenge will be how to measure this normalization in patients. There is a broad debate on the best way of imaging vessels and blood flow; promising results are shown with dynamic MRI, dynamic CT or PET nuclear scanning in specific conditions. The general solution for imaging in clinical studies is not available yet.

It can be hypothesized that revascularization occurs concomitant with tumor growth during treatment with anti-VEGF Rx due to the provoked hypoxia, which in turn results in an upregulation of various angiogenic growth factors (e.g. VEGFA, fibroblast growth factor, ephrins and angiopoietins) which may ultimately result in resistance. Another theory about the mechanism behind revascularization is the mobilization of EPCs derived from the bone marrow, which may subsequently differentiate into mature circulating endothelial cells. Those cells may home to the tumor and form new vessels. Mobilization and recruitment of the circulating endothelial cells can be promoted by VEGFA through interaction with its receptors, FLT1 and KDR, expressed on the endothelial progenitor cells [166]. VEGFA is also thought to promote survival by activating anti-apoptotic pathways in EPCs and CECs [167]. Therefore, VEGF/VEGFR antibodies might have beneficial effects on the outgrowth of tumor vessels.

Tackling the problem of dosing and timing will be difficult because molecular processes in normal and tumor vessels are still not completely understood. It is generally known that the maximal tolerable

dose of anti-VEGF/anti-angiogenic Rx is not always the most optimal dose. The classical phase I and II definitions need to be reset for biological modulators. Moreover, the decision about the most optimal dose is hampered because measurable endpoints are hard to define. Recent studies demonstrated disadvantages of anti-angiogenic/VEGF approaches such as increased trombo-embolic events and hypertension. It still remains to be investigated whether these trombo-embolic events are mainly age, disease and/or treatment related. Also, rational timing of anti-angiogenic/VEGF treatment strategies is difficult due to a lack of complete biological understanding. However, promising results ask for more understanding with global, robust techniques to gain more insights in this process. These insights will guide the way for answering dose and time frame questions.

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Chapter 3

Addition of PTK787/ZK 222584 can lower the dosage of Amsacrine to achieve equal amounts of Acute Myeloid Leukemia cell death

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Abstract

Acute myeloid leukemia (AML) is a disease with a poor prognosis. It has been demonstrated that AML cells express VEGFA and VEGFC as well as KDR (VEGFR2), the main receptor for downstream effects, resulting in an autocrine pathway for cell survival. The present study investigates the role of the VEGFR inhibitor PTK787/ZK 222584 upon leukemic cell death, and the possibility of an additional effect upon cell death achieved by a chemotherapeutic drug Amsacrine. In 3 AML-cell lines and 33 pediatric AML patient samples we performed total cell kill assays to determine the percentage of cell death achieved by PTK787/ZK 222584 and/or Amsacrine. Both drugs induced AML cell death. With a response surface analysis we could show that, in cell lines as well as in primary AML blasts, equal amounts of leukemic cell death can be obtained when lower doses of the more toxic Amsacrine are combined with low dosages of the less toxic VEGFR inhibitor. This study shows that PTK787/ZK 222584 might have more clinical potential in AML when combined with chemotherapy, e.g. Amsacrine. In the future it will be interesting to study whether the complications and long-term effects of chemotherapy can be reduced by lowering dosages of Amsacrine and replace it by other drugs with a lower toxicity profile, such as PTK787/ZK 222584.

Introduction

The outcome of patients with acute myeloid leukemia (AML) has improved due to intensive chemotherapy combined with a reduction in treatment-related mortality. However, the long-term disease-free survival is varying between13-44% dependent on age and cytogenetics so new treatment strategies are still warranted [1-3].

The level of vascular endothelial growth factor A (VEGFA) at time of diagnosis has been described to be an independent prognostic factor for treatment outcome in pediatric AML in a way that high VEGFA levels in AML cells are related to worse outcome [4,5]. VEGFA is the prototype of the VEGF family, a group of angiogenic proteins, and was originally cloned from the HL-60 cell line. It appeared to be a potent stimulator of endothelial cell migration and proliferation [6]. Differential splicing of exon 6 and/or exon 7 results in various isoforms; the isoforms of 121 and 165 amino acids have the most potent activity on endothelial cells [7]. In the meantime, six VEGFs are described; VEGFA to VEGFF [8-12]. These proteins bind with various binding abilities to two cell surface receptor families: the tyrosine kinase receptors (VEGFR) and the neuropilin (NRP) receptors. Three VEGF receptors have been identified, namely VEGF receptor 1 (FLT1), VEGF receptor 2 (KDR) and VEGF receptor 3 (FLT4) [13-15]. Only two neuropilins (NRP1 and NRP2) have been described until now [16,17]. NRP1 appears to act as a coreceptor that enhances VEGF165 binding to KDR on endothelial cells [18].

KDR is thought to be the main receptor for downstream effects of VEGF, and its deletion in mice is lethal [19]. KDR is not only expressed on endothelial cells, in which stimulation enhances proliferation and differentiation of endothelial cells, but is also expressed by AML cells [20]. In response to KDR stimulation intracellular signals are transmitted, such as signaling via mitogen activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K/Akt) cascades, resulting in leukemic cell proliferation and cell survival [22,23]. Anti-apoptotic effects of VEGFC have also been described by FLT4 signaling in AML [24]. The production of VEGFA and VEGFC, as well as VEGFR-expression by AML cells resulted in the knowledge that an autocrine pathway for cell survival exists. Interference with the autocrine VEGF pathway by blockade of VEGF/VEGFR signaling may therefore result in increased apoptosis in leukemic cells.

PTK787/ZK 222584 inhibits the phosphorylation of VEGF receptor tyrosine kinases [25]; it exerts its major effect on KDR (drug concentration needed to inhibit 50% of the receptor, IC_{50} value, 0.037 μM), but it also inhibits FLT1 (IC_{50} 0.077μM), FLT4 (IC_{50} 0.64 μM) and other tyrosine kinases such as the PDGFR-β (IC_{50} 0.58 μM), c-KIT (IC_{50} 0.73 μM) and c-FMS (IC_{50} 1.4μM). The aim of this study was to gain more insight in the effect of PTK787/ZK 222584 upon cell death of AML cell lines as well as primary AML cells. Moreover, the additional effect of the VEGFR inhibition was investigated upon the cell death achieved by a conventional cytostatic drug, e.g. Amsacrine.

Materials and methods

AML cell lines and primary AML blasts

The cell lines HL-60, TF-1, THP-1 and K562 were obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI-1640 medium supplemented with penicillin/streptomycin and 10% fetal bovine serum (FBS, Hyclone, Logan, Utah, USA) for HL-60, THP-1 and K562 cells and additionally supplemented with GM-CSF 1 ng/ml for TF-1. Before incubation with Amsacrine (Pfizer, Capelle a/d IJssel, The Netherlands) and/or PTK787/ZK 222584 (a kind gift of the joint development project between Novartis Pharmaceuticals, Basel, Switzerland and Schering AG, Berlin, Germany). AML cell lines were serum starved overnight in serum free medium. After written informed consent primary AML blast samples at diagnosis from pediatric AML patients were obtained from Dutch Childhood Oncology Group (DCOG), The Hague, the Netherlands, in accordance with the regulations and protocols sanctioned by the medical ethical committee. Diagnosis of AML was confirmed at the laboratory of the DCOG. Table 1 summarizes the patient characteristics. Mononuclear cells were separated by using Lymphoprep (Nycomed, Oslo, Norway) density gradients, cryopreserved in liquid nitrogen until use. Cryopreserved AML cells were thawed rapidly at 37°C, diluted in a 5x volume of normal calf serum (NCS) as described previously [26]. The remaining blast cell population contained >95% AML cells and is referred to hereafter as AML cells.

Table 1. Characteristics of pediatric AML patients.

Characteristics	
No.	33
Sex (male:female), n	22:11
Age at diagnosis, yrs	9 (0 - 16)
Leucocytes at diagnosis, $x10^9$/L	127.6 (7.5 - 355.0)
Thrombocytes at diagnosis, $x10^9$/L	45.0 (7.0 - 221.0)
FAB-classification, n	
M0	5
M1	2
M2	0
M3	0
M4	16
M4eo	2
M5	6
M5A	1
Unknown	1
Died, n	16

The characteristics (age, leucocytes and thrombocytes) are given as median (range). No. indicates the number of patient samples; FAB classification, French American British classification [56].

RNA extraction and Reverse Transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted by Trizol methods following the description of the manufacturer (Life Technologies, Gibco BRL, Grand Island, NY, USA). cDNAs were prepared by reverse transcription at 37°C for at least one hour in a 20 µl reaction mixture containing 2 µg of total RNA, random hexamers (Pfizer, Capelle a/d IJssel, The Netherlands), 5x first strand buffer, RNAsin and reverse transcriptase (Gibco BRL, Grand Island, NY, USA). cDNA was amplified in the presence of primers, 10x buffer, 1.5 mM $MgCl_2$, dNTPs and Taq (Gibco BRL, Grand Island, NY, USA). The mixture was amplified in a Perkin Elmer apparatus with PCR cycle conditions specific for the PCRs tested. The PCR product was analysed by electrophoresis in a 1.5% agarose gel. Gels were stained with ethidium bromide and photographed. Specific primers for β_2-microglobulin were sense (CCA GCA GAG AAT GGA AAG TC) and anti-sense (GAT GCT GCT TAC ATG TCT CG), PCR product: 260 bp; 22 cycli, annealing temperature 55°C. For VEGFA, sense (GAG TGT GTG CCC ACT GAG GAG TCC AAC) and anti-sense (CTC CTG CCC GGC TCA CCG CCT CGG CTT), PCR product: 177, 312, 384 bp; 35 cycli, annealing temperature 55°C, were used. The primers for VEGFA span the splice junctions, enabling the amplified product of splice variants to be separated electrophorically. For VEGFC, sense (AGG CTG GCA ACA TAA CAG AGA A) and anti-sense (TGT AAT TGG TGG GGC AGG TC), PCR product 479 bp; 30 cycli, annealing temperature 65 °C were used.

Fluorescence Activated Cell Sorting

KDR protein expression was measured with the monoclonal anti-VEGF Receptor 2 mouse IgG1 isotype (Sigma, Saint Louis, Missouri, USA) which recognizes an internal epitope of the KDR protein. Cells (0.5 x 10^6) were washed and incubated for 15 min at room temperature, in phosphate buffered saline (PBS). Subsequently, the cells were incubated for 20 min at 4°C with 1.0 µg of monoclonal anti-VEGF Receptor 2 mouse IgG1 isotype or with 1.0 µg IgG1 isotype control (BD Biosciences, San Jose, USA). Cells were washed with PBS and incubated for 20 min at 4°C with PE-conjugated rabbit-anti-mouse F(ab)2 fragments (DakoCytomation, Glostrup, Denmark). PE fluorescence was measured on a FACScalibur flow cytometer and expressed as median fluorescence intensity (MFI). Control cell line used to standardize the KDR protein expression assay included the KDR negative cell line K562.

Cellular drug resistance measurement using a total cell kill assay

For the cell lines Amsacrine (0.001 – 1 µg/ml) at different concentrations, and/or the VEGFR inhibitor PTK787/ZK 222584 (5 -100 µM) at different concentrations, were tested, both in 96-wells micro culture plates, in quadruplicate. 33 patient samples were studied for the effect of the VEGFR inhibitor PTK787/ZK 222584 (5-100 µM), Amsacrine (1 µg/ml) or a combination of those drugs. In 2 patient samples, combined testing was impossible due to a low cell number. In 6 pediatric AML samples more concentrations of Amsacrine (0.001-2 µg/ml) and/or PTK787/ZK 222584 (5-50 µM) in quadruplicate were studied. The pharmacological profile of PTK787/ZK 222584 shows inhibition of the VEGF receptor tyrosine kinases. The strongest inhibition is found against KDR (IC_{50} 0.037 µM); it exhibits a weaker inhibition of FLT1 (IC_{50}

0.077μM) and FLT4 (IC_{50} 0.64 μM). At higher concentrations, the VEGFR inhibitor also inhibits other kinases belonging to the same class as the VEGF receptors, the PDGFR-β, c-kit and c-Fms [27]. The in vitro cellular drug resistance of the cell lines and patient samples (100.000 cells/well) were assessed using a 3-day cell culture assay based on the principle that only viable cells are able to reduce 3-(4,5-dimethylthiazol-2,5-diphenyl) tetrazolium bromide (MTT) (5 mg/ml in PBS) added to each well for 4 hours, to a colored formazan product, measured spectrophotometrically at 520 nm, as described before [28]. The optical density (OD) in the MTT assay is linearly related to the number of viable cells. Control wells contain leukemic cells only with culture media without drugs and blank wells contained culture media only. Percentage of cell survival was calculated at each drug concentration by the equation (mean OD treated wells/mean OD controls wells) x 100% after correction for the background found in the blank wells. The results were considered evaluable when the control wells still contained 70% or more leukemic cells (determined by MGG staining) after 3-day culture period. The mean OD of the control wells after correction for the background at 3 days always exceeded 0.1 arbitrary units for valid results. The LC50 value (drug concentration needed to kill 50% of the leukemic cells) was used to compare the differences between patients and/or various drugs combinations. LC50 value equation: ([% leukemic cell survival > 50%] – 50)/([% leukemic cell survival > 50%] – [% leukemic cell survival < 50%]) x (drug concentration when leukemic cell survival < 50% - drug concentration when leukemic cell survival > 50%) + (drug concentration when leukemic cell survival > 50%) [29].

Statistical analysis

The purpose of the statistical analysis was to study the relation between the (for background activity of media culture corrected) survival (S) and the cytostaticum concentration (CC) and VEGFR inhibitor concentration (VC). For this purpose we used a type of a response surface analysis. The survival proportions S were transformed to logits Y=log[S/(1-S)] and mixed-effects models were fitted to the data [30]. Y was considered as a normally distributed response variable, VC and CC were considered as variables defining fixed effects parameters of the model, and replications entered the model as random intercepts. For patient data the model also included random effects due to patients. The building of a model proceeded as follows. First, we fitted separate models for VC without CC and for CC without VC. These preliminary analyses were used to look for suitable transformations for VC and CC: as simple as possible and such that the model assumptions were satisfied. Based on these preliminary analyses, VC and CC were transformed to V = log(VC+25) and C = log(CC+0.01). For the cell lines HL-60, TF-1 and THP-1 respectively 4, 2 and 4 data points appeared to be outliers - with absolute value of standardized residuals exceeding 3 – and it was decided to exclude these points from the analysis. Fitting mixed-effects model with polynomials in V and C and with their interactions revealed that the data are well described by, for the cell line HL-60 4th order polynomial in V, 3rd order C and 3rd order in product terms, (comprising 15 fixed-effects parameters), for the cell line TF-1 4th order polynomial in both variables and product terms (19 parameters), and for the cell line THP-1 by 3rd order polynomial in both variables and in product terms (13 parameters). For patient cells the best fitting model included 8 fixed-effects parameters. Next

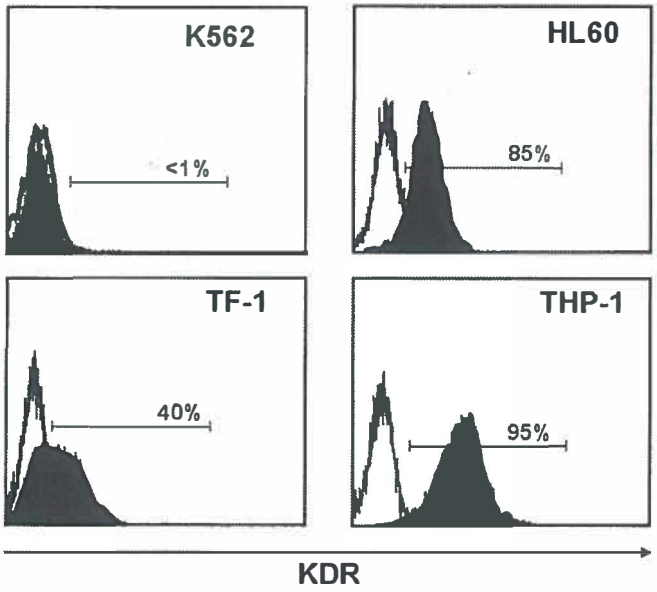
we proceeded to fit joint models in both transformed variables. The best fitting model was used to determine isoboles, i.e. concentration combinations resulting in the same cell survival. Computations were carried out in S-PLUS 6.2 software (Insightful Corporation, Seattle, 2001). The Wilcoxon signed ranks test and the Spearman's rank correlation test were used to compare differences between groups.

Results

In leukemic cell lines the VEGFR inhibitor PTK787/ZK 222584 can partially replace Amsacrine resulting in equal amounts of cell death

To investigate whether cell lines will be potential targets for the VEGFR inhibitor PTK787/ZK 222584 we first determined the expression of VEGFA, VEGFC and KDR. THP-1, TF-1 and HL-60 expressed three splice variants of VEGFA (121, 165 and 189 amino acids). In contrast to VEGFA, only THP-1 cells showed mRNA production of VEGFC (data not shown). Next we determined KDR protein expression (Fig. 1). THP-1 showed the strongest expression of KDR compared to the cell line K562, whereas TF-1 cells were only slightly positive for KDR. PTK787/ZK 222584 is a potent inhibitor of vascular endothelial growth factor receptor tyrosine kinases [31]; it inhibits the VEGF-induced phosphorylation and its action can be abolished by addition of VEGFA. PTK787/ZK 222584 is most potent against KDR, but has also an effect on FLT1 and FLT4.

Figure 1. FACS analysis of the KDR expression in the leukemic cell lines TF-1, THP-1, HL-60 and K562.



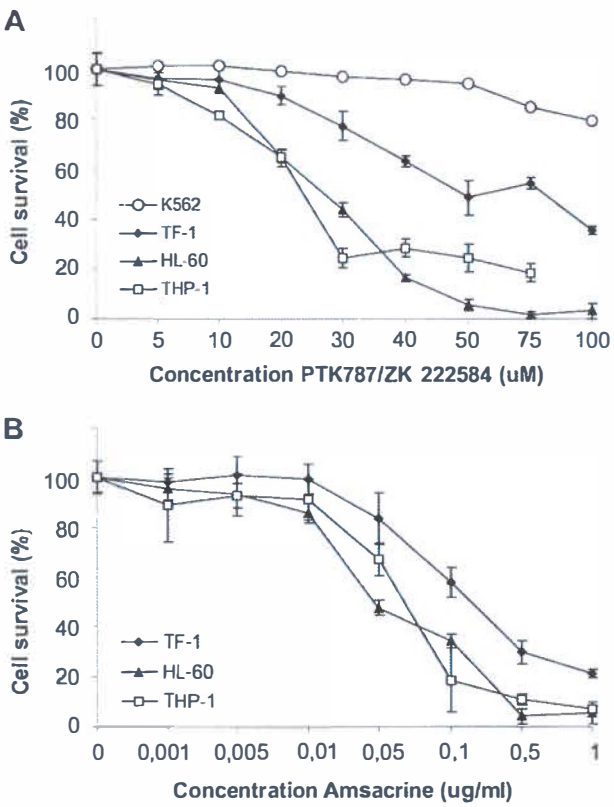
THP-1 showed the strongest expression of KDR compared to the negative control cell line K562. HL-60 showed a weaker expression whereas TF-1 cells were only slightly positive for KDR.

Direct effects of PTK787/ZK 222584 on the four leukemic cell lines were determined. In a total cell kill assay we demonstrated that the VEGFR inhibitor induces cell death in all three cell lines, as shown in Figure 2A (LC50 values for HL-60: 27 µM, TF-1: 49 µM, THP-1: 24 µM). An excellent cell survival was seen on the KDR negative cell line K562 demonstrating that the cell death induced by PTK787/ZK 222584 at identical concentrations is not a toxic effect.

As shown before by others phosphorylation of KDR was decreased dose dependently when the inhibitor PTK787/ZK 222584 was used in our system (data not shown) [32].

The topoisomerase inhibitor Amsacrine is one of the drugs used in AML treatment. For this experiment Amsacrine was chosen because of reproducible and moderate cell death results in all cell lines; with a drug resulting in hardly 50 percent of cell death at full dose, as well as with a drug resulting in massive cell death at the lowest concentration, it will be hard to show additional effect of other agents in vitro.

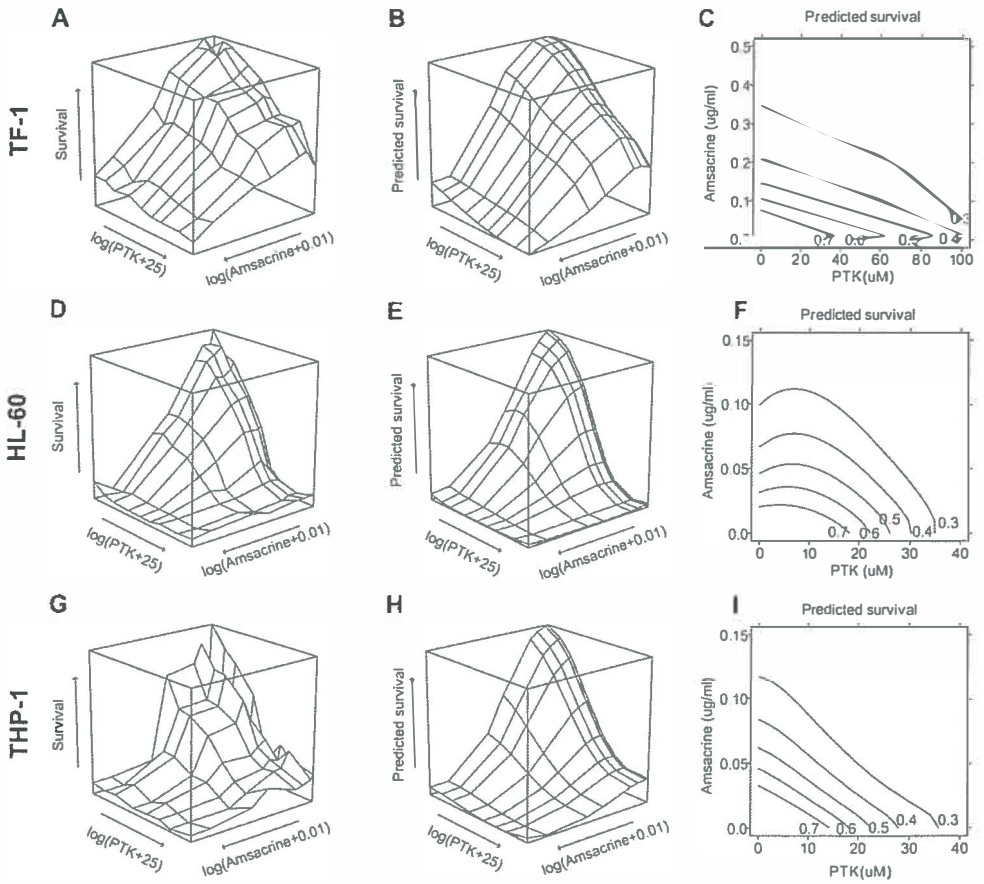
Figure 2. Cell survival percentages after incubation with the VEGFR inhibitor PTK787/ZK 222584 or Amsacrine, determined by using a MTT assay.



(**A**) The data indicates that the VEGFR inhibitor induces cell death in all three cell lines. HL-60 showed the strongest response, whereas TF-1 is least sensitive to the VEGFR inhibitor. Values are expressed as means; bars SE. (**B**) The data clearly indicates that Amsacrine induces cell death in all three cell lines. Values are expressed as means; bars SE.

Leukemic cell survival after incubation with various concentrations of Amsacrine is shown in Figure 2B. Our results clearly point out that all three cell lines were sensitive to Amsacrine with LC50 values for HL-60 0.047 µg/ml, TF-1 0.207 µg/ml and THP-1 0.067 µg/ml.

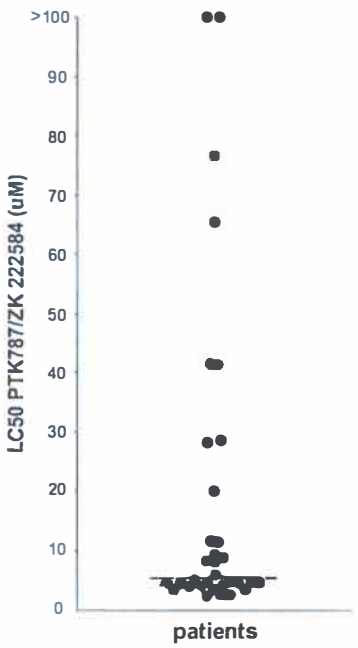
Figure 3. Interaction data between the VEGFR inhibitor PTK787/ZK 222584 (PTK) and Amsacrine for the three AML cell lines.



(**A, D, G**) 3D-plots presenting the raw data for each cell line. (**B, E, H**) 3D-plots presenting a predicted model based on the raw date, demonstrated for all three cell lines. In these six figures the vertical axis represents the (predicted) survival of the cell line, estimated at different concentrations of both drugs (horizontal axes). These six plots show that increasing the concentration of one or both drugs (in the figures named log Amsacrine and log PTK) results in a decrease of the survival. (**C, F, I**) A model that can be used to calculate drug combinations which result in a predicted survival, so called isoboles. The lines in these figures (shown as 0.3 up to 0.7) represent the drug combinations at which 30%, 40%, 50%, 60% or 70% of cell survival will be achieved. All three figures point out that the concentration of Amsacrine can be lowered and replaced by a dose of the VEGFR inhibitor PTK787/ZK 222584 to achieve the same cell death percentage. In the 3D-plots the (transformed) PTK and Amsacrine axes extend over the full range of values as used in the experiments, respectively 0 to 50 and 0 to 2. The scale used in the isobole plots differs per cell line.

To investigate the additional effect of VEGFR inhibition upon cell death achieved by Amsacrine, we combined both drugs in a total cell kill assay. A response surface analysis was used, which emphasizes on finding a particular treatment combination which causes maximum or minimum responses. Figure 3 presents plots of cell survival as a function of concentrations of both drugs. For each cell line the raw data are displayed in the first plot (Fig. 3A, D, G). The raw data are transformed into predicted survival models, based on the best fitting models for the polynomials and parameters (Fig. 3B, E, H). The raw data and the model predicted survival demonstrate for each cell line that when increasing the concentration of one or both drugs the survival percentage of the AML cells will decrease. Next, the best fitting model was used to determine isoboles (Fig. 3C, F, I). The isoboles estimate the drug combinations resulting in the same survival; the lines in the Figure represent the drug combinations at which 30%, 40%, 50%, 60% or 70% (0.3 up to 0.7) cell survival will be achieved. For example, for TF-1 Figure 3C suggests that to achieve a 50% leukemic cell survival (the line 0.5) this can be the result of Amsacrine alone dosed at 0.15 µg/ml, or Amsacrine at 0.12 µg/ml combined with 20 µM PTK, or 0.03 µg/ml Amsacrine combined with 80 µM PTK. Interestingly, the isoboles for each cell line point out that the concentration of Amsacrine can be lowered by a certain dose of the potentially less toxic VEGFR inhibitor PTK787/ZK 222584 to achieve the same percentage of leukemic cell death.

Figure 4. The LC50 value of 33 pediatric AML samples.



The concentration needed to kill 50 percent of the cells after incubation with the VEGFR inhibitor PTK787/ZK 222584. The bar represents the median value of 5.1 µM. In two primary blast samples the LC50 value was above 100 µM PTK787/ZK 222584. Patients with a LC50 value above the median did not differ from patients with a LC50 value below the median regarding age at diagnosis, sex, FAB classification or WBC count.

PTK787/ZK 222584 increases cell death in primary leukemic blasts when combined with Amsacrine

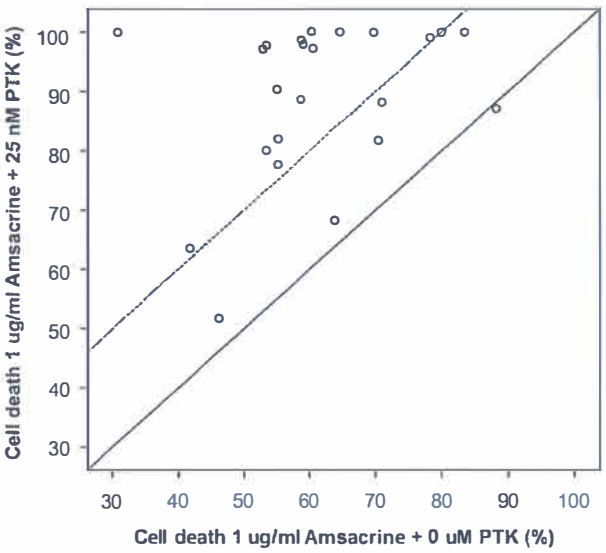
Underscoring the results in the literature, KDR expression was demonstrated in all AML samples tested [33]. Figure 4 shows the LC50 values of the primary AML samples with a median of 5.1 µM (n=33). Primary blast samples are overall 5-10 fold more sensitive to PTK787/ZK 222584 than the leukemic cell lines used in this study. Patients with a LC50 value above the median did not differ from patients with a LC50 value below the median regarding age at diagnosis, sex, FAB classification, leukocyte count or thrombocyte count. Moreover, there was no relation between the expression of KDR in the patients we tested and the sensitivity to PTK787/ZK 222584 (Spearman's rho=-0.63, p=0.825).

The next step was to test the addition of the VEGFR inhibitor PTK787/ZK 222584 to Amsacrine on the primary AML blasts and to determine the cell death percentage induced by both drugs (n=31). We first treated the patient samples with 1 µg/ml Amsacrine alone; we chose 1 µg/ml Amsacrine because this concentration was near the LC50 value of the patient samples. A varying percentage of cell death was seen for 1 µg/ml Amsacrine alone, with a median value of 60.6%. Interestingly, we found a correlation between the LC50 values of the VEGFR inhibitor PTK787/ZK 222584 and the cell death percentages induced by 1 µg/ml Amsacrine (Spearman's rho=-0.386, p=0.032), which means that samples sensitive to one drug are also sensitive to the other drug. Two samples achieved a cell death percentage of >90% and were excluded from further combination therapy because it is not possible to show additional effect of the VEGFR inhibitor PTK787/ZK 222584.

Next 29 patient samples were treated with 1 µg/ml Amsacrine and 25 µM PTK787/ZK 222584, with a median value of 92.3% cell death. Figure 5 demonstrates the cell death percentage achieved by 1 µg/ml Amsacrine with 25 µM PTK787/ZK 222584 compared to the cell death percentage achieved by 1 µg/ml Amsacrine alone. Addition of 25 µM PTK787/ZK 222584 resulted in an increase in cell death of >20% (median increase in cell death 24%) in 18 of the 29 samples (62%), whereas 10 samples showed a moderate increase between 0 – 20%. No significant additional cell death was demonstrated when PTK787/ZK 222584 dosage was increased up to 100 µM.

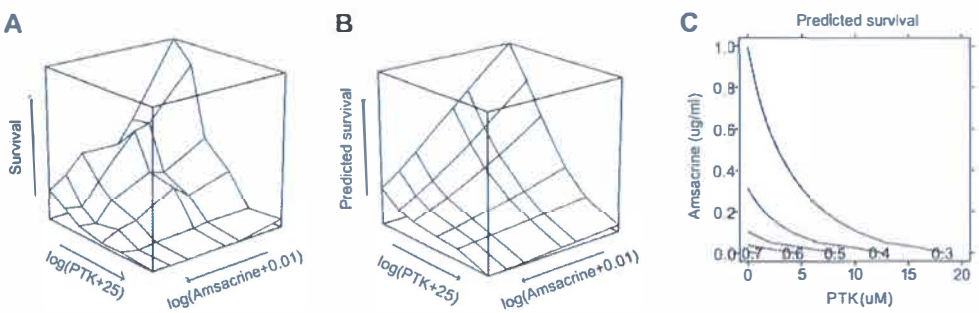
In addition, for 6 AML patient samples the combination of Amsacrine and PTK787/ZK 222584 treatment was investigated more extensively to obtain isoboles for AML patient samples. The interaction between PTK787/ZK 222584 and Amsacrine for primary AML blasts is given in Figure 6. Again in Figure 6A the raw survival data are demonstrated, whereas Figure 6B represents the predicted model upon the raw data. In Figure 6C the isoboles are shown. Identical to the cell line results, also in primary patient material we were able to demonstrate that lowering the concentration of the chemotherapeutic drug Amsacrine resulted in identical cell death by adding a certain dose of the potentially less toxic VEGFR inhibitor PTK787/ZK 222584 to primary AML blasts.

Figure 5. The cell death percentage achieved by 1 µg/ml Amsacrine combined with 25 µM PTK787/ZK 222584 compared to the cell death percentage achieved by 1 µg/ml Amsacrine alone.



The solid line represents equal amounts of cell death with Amsacrine ± PTK787/ZK 222584, whereas the dashed line represents 20% increase in cell death with addition of PTK787/ZK 222584 ($p < 0.001$, Wilcoxon signed ranks test). The samples above the dashed line achieved an increase in cell death of >20% when treated with 25 µM PTK787/ZK 222584 added to 1 µg/ml Amsacrine; 18 of the 29 samples showed an increase in cell death of >20%.

Figure 6. Interaction data between the VEGFR inhibitor PTK787/ZK 222584 (PTK) and Amsacrine for primary AML blasts (n=6).



(**A**) 3D-plots presenting the raw data in terms of survival and logits for patient samples. (**B**) 3D-plots presenting a predicted model for logits and survival demonstrated for patient samples. The vertical axes in these figures represent the (predicted) survival of the patient samples, estimated at different concentrations of both drugs (horizontal axes). Increasing the concentration of one or both drugs results in a decrease of survival. (**C**) An isobole of the patient samples. This figures contains lines (shown as 0.3 up to 0.7), which represent the drug combination at which 30%, 40 %, 50%, 60% or 70% of cell survival will be achieved. All three figures point out that the concentration of Amsacrine can be lowered and replaced by a dose of the VEGFR inhibitor PTK787/ZK 222584 to achieve the same cell death percentage.

Discussion

The results of the present study show that PTK787/ZK 222584, a VEGFR inhibitor, has potent in vitro cell death effects against three AML cell lines and primary AML blasts. Interestingly, we show that in a combined strategy the toxic Amsacrine can be partially replaced by a potentially less toxic VEGFR inhibitor, PTK787/ZK 222584, inducing equal amounts of leukemic cell death in vitro, not only in cell lines but also in primary AML blasts.

VEGF/ VEGFR signaling has been described in several tumors, such as multiple myeloma [34], melanoma [35] and leukemia [36], resulting in proliferation and survival in tumor cells. In our study we demonstrate that HL-60, THP-1 and TF-1 do show expression of KDR and respond to the VEGFR inhibitor PTK787/ ZK 222584. The low sensitivity of TF-1 might be the result of a low KDR expression. The KDR negative cell line K562 showed no response to the VEGFR inhibitor PTK787/ZK 222584, whereas K562 is positive for FLT1 and FLT4 [37,38]. In all patient samples we demonstrated high KDR expression, supporting recent results of others [39]. A relation between the expression of KDR and the sensitivity for PTK787/ZK 222584 was not found.

Recently, studies of other VEGFR inhibitors for the treatment of AML have been published. SU5416, a Receptor Tyrosine Kinase (RTK) inhibitor of c-kit, FLT3 and KDR (IC_{50} 0.20 μM) induces growth arrest and apoptosis in AML cells in vitro [40,41]. A phase 2 study of SU5416 in refractory AML showed modest clinical activity; in 17 of 42 patients, despite continuous therapy, a strong increased blast count was found [42]. Poor oral bioavailability might be a reason for these results [43]. Interestingly, in this particular study patients with AML blasts expressing high levels of VEGF had a significantly higher response rate than patients with low VEGF expression.

Furthermore, a phase 1 study of SU11248, RTK inhibitor of c-Kit, FLT3, KDR (IC_{50} 10 nM) and PDGFR-β in the treatment of patients with refractory of resistant AML showed molecular and clinical activity in AML [44]. The inhibitor induced partial remission of short duration.

Two phase 1 trials with PTK787/ZK 222584 treatment in patients with advanced colorectal cancer show that patients with stable disease for at least 2 months achieve up to 40% tumor regression [45]. The first results from a phase 3 study in patients with metastatic adenocarcinoma of the colon or rectum demonstrate that patients who receive a combination of PTK787/ZK 222584 with a chemotherapy regimen had a 17 percent reduction in risk of disease progression compared to chemotherapy treatment alone [46]. Further analyses on the data including information on the overall survival points are expected soon.

In a phase 1 study of PTK787/ZK 222584 for the treatment of primary refractory or relapsed AML, no significant responses to treatment with PTK787/ZK 222584 were found. PTK787/ZK 222584 was also tested in patients with secondary AML; monotherapy resulted in a stable disease for 10-14 months in 2 of the 35 patients. Of 17 patients treated with induction chemotherapy and PTK787/ZK 222584, five patients achieved CR [47]. The potential clinical activity of PTK787/ZK 222584 in AML cell lines warrants

further clinical investigations.
It is demonstrated that single agent VEGFR inhibitor resulted in initial response, however with marginal duration. These results suggest ongoing activation by other signaling pathways. Recently, Kornblau et al showed that activation of multiple signaling transduction pathways in AML patients is common. The prognosis of AML patients worsens if more signaling transduction pathways are activated [48]. Therefore, targeting more signaling pathways by conventional chemotherapeutic drugs and/or specialized tyrosine kinase inhibitors might be beneficial for patients.
Interestingly, another clinical approach for the treatment of AML was chosen by using Bevacizumab, an anti-VEGFA monoclonal antibody following chemotherapy [49]. The administration of Bevacizumab targets extracellular VEGFA and thereby prevents the stimulation resulting from VEGFA binding to cell surface receptor tyrosine kinases. In adults with relapsed and refractory AML that are resistant to traditional chemotherapy, combining cytotoxic chemotherapy followed by Bevacizumab resulted in a 33% complete remission rate and a median disease free survival of 7 months in 35% of the patients. In summary, all these clinical studies show that there is a possible therapeutic role blocking the VEGF/VEGFR signaling in treatment of AML in vivo when combined with chemotherapy.

An internal and external autocrine VEGF/VEGFR pathway is demonstrated to regulate VEGFR signaling [50,51]. External/paracrine VEGF stimulation of hematopoietic stem cells (HSCs) did not result in survival or proliferation, but blocking the internal loop with intracellularly acting inhibitors of VEGFR dramatically reduced colony formation of HSCs, demonstrating the importance of a VEGF/VEGFR internal/autocrine loop on these cells. In contrast, in AML patient samples and leukemic cell lines it is shown that treatment with an external blocker resulted in a shift in KDR localization from the nucleus to the surface of the cell suggesting that VEGF needs to be exported to internalize KDR and activate signaling pathways. Moreover, internal and external inhibitors exerted their effect via distinct mechanisms, the internal having the strongest pro-apoptotic effects by affecting the MAPK/ERK and the PI3K/AKT pathways. Santos et al also found that cells treated with both the internal and external VEGF/KDR blocker in vitro undergo cell death by apoptosis, even significantly to a greater extent than either drug alone. Another interesting difference in these studies is that KDR is thought to be the main receptor for leukemic cell growth and cell survival, whereas stimulation of both FLT1 and KDR is able to rescue survival of VEGF-deficient HSCs. PTK787/ZK 222584 exerts its effect on KDR and, to a lesser extent, on FLT1, but targeting both the internal and external pathway might even induce more cell death.

In this study we show that combined use of PTK787/ZK 222584 and Amsacrine results in cell death and that in a combination strategy a low dose of Amsacrine combined with PTK787/ZK 222584 can result in identical cell death percentages in vitro as a high dose Amsacrine alone. Whereas identical results will be obtained in vivo remains to be investigated. In vivo, VEGF/VEGFR signaling results not only in autocrine effects in AML cells, but also induces effects in the surrounding tissues. For instance, VEGFR inhibitors might inhibit paracrine effects in which VEGFR stimulation of stromal bone marrow cells results in

interleukin 6 (IL6) and G-CSF, known as potential AML growth factors [52]. Moreover endocrine effects can be inhibited in which VEGF induces new vessel formation; a crucial prerequisite in tumor growth [53].

Amsacrine is a DNA topoisomerase inhibitor; DNA topoisomerase inhibitors were shown to transactivate the vascular endothelial growth factor promoter resulting in the expression of VEGF. It is known from literature that topoisomerase inhibitors can cause treatment related AMLs because these drugs induce chromosomal breakage [54]. Moreover, a lot of chemotherapeutic agents, e.g. Amsacrine, are also known for their cardiotoxicity [55]. To reduce the risk and complications of long-term effects it would be better to find a treatment strategy in which the doses of those chemotherapeutic drugs, for instance Amsacrine, can be at least lowered.

Acknowledgements

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Chapter 4

Impaired long-term expansion and self-renewal potential of pediatric Acute Myeloid Leukemia Initiating Cells by PTK787/ZK 222584

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Submitted.

Abstract

Although most children with Acute Myeloid Leukemia (AML) achieve Complete Remission, the relapse rate is 30-40%. Since it is thought that Leukemia Initiating Cells (LICs) are responsible for AML relapses, targeting these cells might improve outcome. Treatment of pediatric AML blasts with the receptor tyrosine kinase (RTK) inhibitor PTK787/ZK 222584 (PTK/ZK) induces cell death *in vitro*. However, the role of PTK/ZK inhibition on outgrowth of (pediatric) LICs is unknown. In this study we cultured CD34+ cells from pediatric AML patients on MS5 stromal cells in long-term cocultures. In analogy to adult AML, long-term expansion of leukemic cells up to 10 weeks could be generated in 9 of 13 pediatric AMLs. Addition of PTK/ZK to long-term cocultures significantly inhibited leukemic expansion in all samples, ranging from 4-80% growth inhibition at week 5 compared with untreated samples. In 75% of the samples the inhibitory effect was more pronounced at week 10. Proteome profiler array analysis of downstream kinases revealed that PTK/ZK reduced activation of PI3K/Akt kinase signaling. Although main targets of PTK/ZK are Vascular Endothelial Growth Factor (VEGF) receptors, no effect was seen on outgrowth of LICs when exposed to stroma-derived VEGFA or cultured with Bevacizumab (a monoclonal VEGFA antibody). These data suggest that the effect of PTK/ZK on LICs is not only dependent on inhibition of VEGFA/VEGFR signaling. Taken together, our data elucidated anti-leukemic properties of PTK in long-term expansion cultures, and suggest that targeting multiple RTKs by PTK/ZK might be a potential effective approach in eradicating (pediatric) LICs.

Introduction

Children with Acute Myeloid Leukemia (AML) have a moderate to poor prognosis. Despite an initial Complete Remission (CR) rate of 90%, 30-40 % of the pediatric AML patients relapse, and the 5-year Overall Survival rate is approximately 60% [1-3]. Therefore, certain leukemic cells have to be resistant to current treatment strategies, and persist after therapy. AML is a hematological disease, which may originate at the level of the pluripotent hematopoietic stem cell [4]. It was thought that the leukemic blast population is organized as a hierarchy, whereby LICs (also known as Leukemic Stem Cells) reside at the top of this hierarchy [5,6]. Since therapy-surviving LICs may be responsible for AML relapses, it seems advantageous to attack and eradicate these cells in order to improve survival rates.

It has been suggested that LICs depend on bone marrow niches for self-renewal, like Hematopoietic Stem Cells (HSCs) [7]. HSCs reside within specialized 'stem cell niches' in the bone marrow, and these niches have been defined by their secretion of specific signaling molecules, growth factors, and cytokines including macrophage colony-stimulating factor (MCSF), interleukins, stem cell factor (SCF, KIT-ligand), and vascular endothelial growth factor A (VEGFA) [8-11]. Interaction with the cytokines dictates, at least in part, stem cell fate, indicating an important role for the environment-derived cytokines and its receptors [7,12].

PTK787/ZK 222584 (PTK/ZK) is a small-molecule kinase inhibitor that penetrates into cells and reaches its intracellular target, with as a result a decrease in phosphorylation of the VEGF RTKs VEGFR2 (kinase insert domain receptor (KDR), IC50 0.037 μM) and VEGFR1 (fms-related tyrosine kinase 1 (FLT1), IC50 0.077 μM). At higher concentrations it also inhibits other RTKs including platelet-derived growth factor receptor β (PDGFRβ, IC50 0.58 μM), VEGFR3 (fms-related tyrosine kinase 4 (FLT4), IC50 0.64 μM), c-KIT (KIT, also known as CD117 or SCFR, IC50 0.73 μM) and colony-stimulating factor-1 receptor (CSFR1, also known as c-FMS, IC50 1.4 μM) [13](10). We previously showed that PTK/ZK induces a dose-dependent decrease in cell survival in three AML cell lines as well as in 33 primary pediatric AML blasts in short term cultures [14]. The level of VEGF at time of diagnosis has been shown to be an independent prognostic factor for biological response in (pediatric) AML, e.g. occurrence of relapse [15-18]. AML cells can produce VEGF and express its receptors, which make AML cells sensitive for VEGF dependent proliferation [19]. The downstream effects of VEGF are mainly executed by VEGFR2 binding, resulting in increased AML cell survival and proliferation via downstream signaling pathways such as via mitogen activated protein kinase (MAPK) or the phosphatidylinositol 3-kinase (PI3K) pathway [20,21]. However, these downstream signaling pathways can also be activated by various other RTKs, including c-KIT and PDGFR-β [22-24].

In this study we have investigated the role of the tyrosine kinase inhibitor PTK/ZK on the (out)growth and self-renewal of pediatric LICs. To study the interaction between bone marrow derived stromal cells and LICs, a previously described leukemic long-term culture initiating cells (LTC-IC) assay has been used in which long-term leukemic expansion of LICs can be established using MS5 bone marrow stromal

cells, thereby mimicking the stem cell niche [25]. We cultured sorted CD34+ pediatric AML cells on stroma in the absence or presence of PTK/ZK. Our results indicate that PTK/ZK induced a decrease in long-term AML expansion.

Material and Methods

Establishing leukemia long-term cultures on stroma

AML blasts from peripheral blood cells or bone marrow cells from untreated pediatric AML patients were studied after informed consent. AML mononuclear cells were isolated by density gradient centrifugation and $CD34^+$ cells were selected by MoFlo sorting (DAKO Cytomation, AS, Glostrup, Denmark). Sort efficiency was measured by FACS analysis and CD34+ percentages of 95-99% were found. 40-50 x 10^3 sorted CD34+ cells (i.e. LICs) were plated in 12-well plates pre-coated with confluent layer of MS5 stromal cells. Cells were expanded in LTC medium (alphaMEM supplemented with heat-inactivated 12.5% FCS, heat-inactivated 12.5% horse serum (Sigma, Zwijndrecht, the Netherlands), penicillin and streptomycin, 2 mM Glutamine, 57.2 µM beta-mercaptoethanol (Sigma) and 1 µM hydrocortisone (Sigma)) supplemented with 20 ng/ml IL-3, G-CSF and TPO as previously described [25]. Cultures were kept at 37°C and 5% CO_2. Cultures were demidepopulated weekly for analysis. Self-renewal was studied in cocultures that generated Leukemic Cobblestone Areas (L-CAs) by harvesting suspension and adherent cells at week 5, and CD45+ cells were sorted and replated onto new MS5 stroma to initiate 2nd cocultures. Cells were cultured in the presence of 10 µM PTK787/ZK 222584 (a kind gift from the joint development project between Novartis Pharmaceuticals, Basel, Switzerland and Schering AG, Berlin, Germany) or 25 µg/ml Bevacizumab (a kind gift from Genentech/Roche). Used concentration of PTK/ZK based on previously results, used concentration Bevacizumab based on literature [14,26]. DMSO was used as a control. All drugs were added during demidepopulation. The fact that cocultures generated L-CAs after replating, a feature of self-renewal that has not been demonstrated in normal cord blood CD34+ cells, confirms the leukemic origin of the expanding cells [25]. In addition, with FLT3-ITD fragment analysis of the suspension cells in the LTC-IC co-cultures at week 2, we demonstrated for AML10 (FLT-ITD positive sample) that the suspension cells harbor the heterozygous FLT3-ITD mutation (86%).

AML cell lines THP-1 and MOLM13

The cell lines THP-1 and MOLM13 were obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI-1640 medium supplemented with 1% penicillin/streptomycin (Sigma Aldrich) and 10% fetal bovine serum (FBS, Hyclone, Logan, Utah, USA).

Transduction of MS5 stromal cells

MS5 cells were cultured in alpha-MEM medium supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS, Hyclone, Logan, Utah, USA). Retroviral supernatants were generated by

cotransfection of 2 µg reporter constructs pMSCV-iGFP-VEGFA165 or pMSCV-iGFP (empty vector, negative control) and 2 µg packaging plasmid pCLampho into 293T cells using FuGENE HD transfection reagent (Roche, Almere, the Netherlands). 5×10^4 MS5 cells were incubated with retroviral supernatants, which were filtered through 0.45-µm pore size syringe-mounted filters. Incubation was supplemented with 8 µg/ml polybrene. This procedure was repeated for two consecutive days after which stably transduced cells were expanded. Transduction efficiency was measured by FACS analysis, which demonstrated an efficiency of 94% for cells transduced with the empty vector (MS5-control) and 81% for cells transduced with VEGFA165 (MS5-VEGFA). Cells were sorted on a MoFlo.

RNA Extraction and Real-Time PCR

Total RNA from MS5 transduced cells or pediatric AML cells (material from 11 AML samples at start of the culture was available) were extracted with NucleoSpin RNA II kit according to manufacturer's protocol (Macherey-Nagel, Duren, Germany). cDNA was prepared at 37°C for at least one hour in 20 µl reaction mixture containing 2 µg of total RNA, random hexamers (Pfizer, Capelle a/d IJssel, The Netherlands), 5x first strand buffer, RNAsin and reverse transcriptase (Gibco BRL, Grand Island, NY, USA). Real-time PCR was performed using iQ SYBR green supermix (Bio-Rad, Hercules, CA, USA). All PCR reactions and data analysis were performed on the iCycler iQ Real-Time Detection System (Bio-Rad). Specific primers are shown in Supplementary Table S1. The expression of the RTKs and VEGFA was standardized for expression of β-actin and/or RPL22 (Arbitrary Units, AU).

ELISA and functional assay of VEGFA

Secretion of VEGFA was detected in supernatant using commercially available ELISAs (Quantikine immunoassays, R&D systems, Abingdon, United Kingdom) following manufacture's instructions. The functionality of secreted VEGFA from transduced cells was detected by adding its supernatant to endothelial cells (HUVECs) and quantify expression of VEGFA specific genes EGR3, NUR77 and NOR1 in endothelial cells with real-time PCR, described in detail by Liu et al [27] (Supplementary Figure S1).

Microscopy and cytospins

For morphological analysis, May-Grünwald Giemsa staining was used to analyze cytospins. Pictures of MS5 cocultures and cytopspins were taken at magnification 400x. Morphological analysis of May-Grunwald-Giemsa stains showed an immature myeloid phenotype after at least four weeks of culture.

Flow cytometry analysis

Cells were blocked by PBS 1% BSA (Bovine Serum Albumin, Sigma), and stained with anti-VEGFR1 (Sigma Aldrich), anti-VEGFR2/KDR antibody (Sigma Aldrich), anti-VEGFR3/FLT4-APC (R&D systems), anti-CD115/cFMS-biotin (R&D systems), anti-CD34-PE, anti-CD38-PerCP-Cy5.5, anti-CD117-PerCP, anti-CD140b/PDGFRβ-PE (BD Bioscience). Primary VEGFR1, and KDR antibodies were visualized using PE-conjugated

secondary antibody (Dako cytomation). Primary CD115 antibodies were visualized using streptavidine FITC. IgG1-FITC/ PE/APC/PerCP were used as a negative isotype controls. THP-1 and MOLM13 cells were stained with Annexin V-FITC and PI for 15 minutes in staining buffer following manufacturer's protocol (Annexin-V-FLUOS staining kit, Roche). Necrotic, early apoptotic, and viable populations are distinguished based upon Annexin V and PI expression. Expression was analyzed using LSRII (BD FACS DIVA software, BD bioscience). The data was eventually developed using FlowJo software (Tree Star Inc., Ashland, Oregon, USA). Expression percentages ≥3% were considered as actual protein expression, above isotype controls.

Phosphokinase arrays

Proteome Profiler Human Phospho-Kinase Array kits from R&D Systems, Inc. (Minneapolis, MN, USA) were used to measure protein phosphorylation according to protocol. Per patient sample, 50µg protein was applied to the array. In short, in this method proteins are captured by antibodies spotted on a nitrocellulose membrane. Levels of phosphoprotein are then assessed using a horseradish peroxidase (HRP)-conjugated antibody followed by chemiluminescence detection. In our experiment, the amount of chemiluminescence was detected and analyzed using array software (ScanAlyze; Eisen Software, http://rana.lbl.gov/eisen).

Statistical analysis

Statistical differences in leukemic outgrowth between co-cultures were determined in a paired Wilcoxon signed rank test.

Results

Long-term culture of pediatric LICs on MS5 bone marrow stroma

Culture of sorted CD34+ pediatric AML cells in an LTC-IC assay showed a long-term expansion of leukemic cells up to 10 weeks in 9 out of 13 AMLs (Fig. 1A, patient characteristics listed in Table 1). Expansion of LICs on MS5 stromal cells was associated with the formation of phase-dark Leukemic Cobblestone Areas (L-CAs) underneath the stroma, appearing after 2 to 5 weeks of culture (Fig. 1B). Self-renewal capacity of the pediatric AML cells could be demonstrated by initiating secondary co-cultures on new MS5 stroma after 5 weeks of culture. Secondary L-CAs were formed in 8 of the 9 cases cultured up to 10 weeks. These data show that a long-term culture of the primitive subfraction of pediatric AML cells can be established at least up to 10 weeks. In contrast, sorted CD34- pediatric AML cells were not able to initiate long-term expansion or secondary co-cultures (n=4, samples AML2, AML5, AML6, and AML10, data not shown).

Table 1. Patient characteristics.

Patient ID	BM/PB	FAB	Karyotype	Growth up to wk	Experiment	%CD34+ cells
AML1	BM	M2	NK	10	P/M	28,7
AML2	BM	M4	inv(16)	7, then †	P/B	68,8
AML3	BM	M0	9q-	10	P/M	83,1
AML4	BM	M3	t(15;17)	9, then †	P/M	16,8
AML5	BM	M3	t(15;17)	10	P/B	2,3
AML6	PB	M5	complex	10	P	0,5
AML7	BM	M0	complex	10	P/M	88
AML8	PB	M1	NK	8, then †	P/M	15,2
AML9	BM	M4	NK	10	P/M	48,4
AML10	BM	M2	t(6;9)	10	P/B	28,6
AML11	BM	M2	t(8;21)	10	P/B	77,9
AML12	BM	x	NK	9, then †	B	61,1
AML13	BM	M5	+8	10	B	0,8

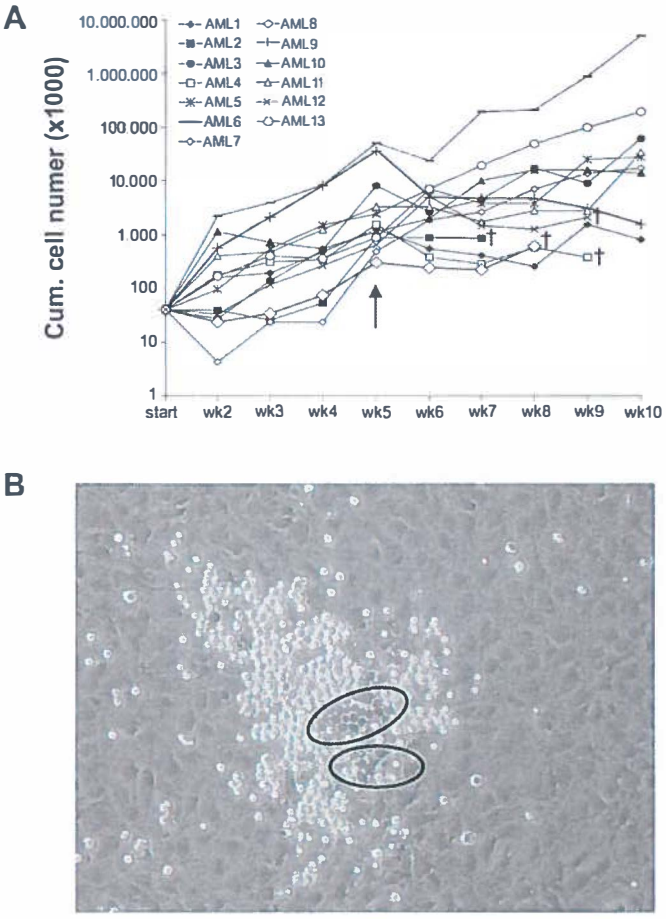
CD34+ cells were isolated from bone marrow (BM) or peripheral blood (PB); percentage CD34+ cells in the total AML mononuclear cell fraction; AMLs were categorized according to the French-American-British (FAB) classification; Karyotype is indicated; experiment performed with PTK/ZK (P) and/or Bevacizumab (B), and/or cultured on MS5-control/MS5-VEGFA stromal cells (M). x Not Available.

Impaired outgrowth and self-renewal of pediatric LICs by the tyrosine kinase inhibitor PTK/ZK

We previously reported that PTK/ZK induces cell death in leukemic cell lines as well as in primary pediatric AML samples. PTK/ZK increased the percentage of (early) apoptotic cells in a dose-dependent way in THP1 and MOLM13 cell lines (high respectively low VEGFR expression) (Supplementary Fig. S1A-B). In primary AML cells PTK/ZK at a concentration of 10μM was effective in reducing AML cell survival [14]. To investigate the effect of PTK/ZK on long-term expansion and proliferation, CD34+ sorted pediatric AML cells were cultured in the absence or presence of 10 μM PTK/ZK in the LTC-IC assay (n=11). Figure 2 represents the growth curves of 11 individual patients and demonstrates that addition of PTK/ZK led to a decrease in leukemic expansion in all cases. The median value of 11 samples showed a significant ($P<0.05$) delay in expansion throughout week 5 to 10. However, variability in sensitivity of the cultured AMLs for PTK/ZK was shown by a decrease in growth, ranging from 4-80 % at week 5 as compared with the controls. After 10 weeks of culture, the inhibitory effect of PTK/ZK became even more pronounced in 75% of the samples (Table 2). Self-renewal potential was inhibited by PTK/ZK as shown by the reduced capability to initiate secondary cocultures after replating (Table 2). Analysis of May-Grunwald-Giemsa stains showed an enhanced differentiation in PTK/ZK treated cells compared with the control cells. Control AML cells of AML5 and AML11 showed a less mature phenotype, whereas the treated AML cells displayed a more differentiating phenotype (Fig. 3A), suggesting that treatment with PTK/ZK might

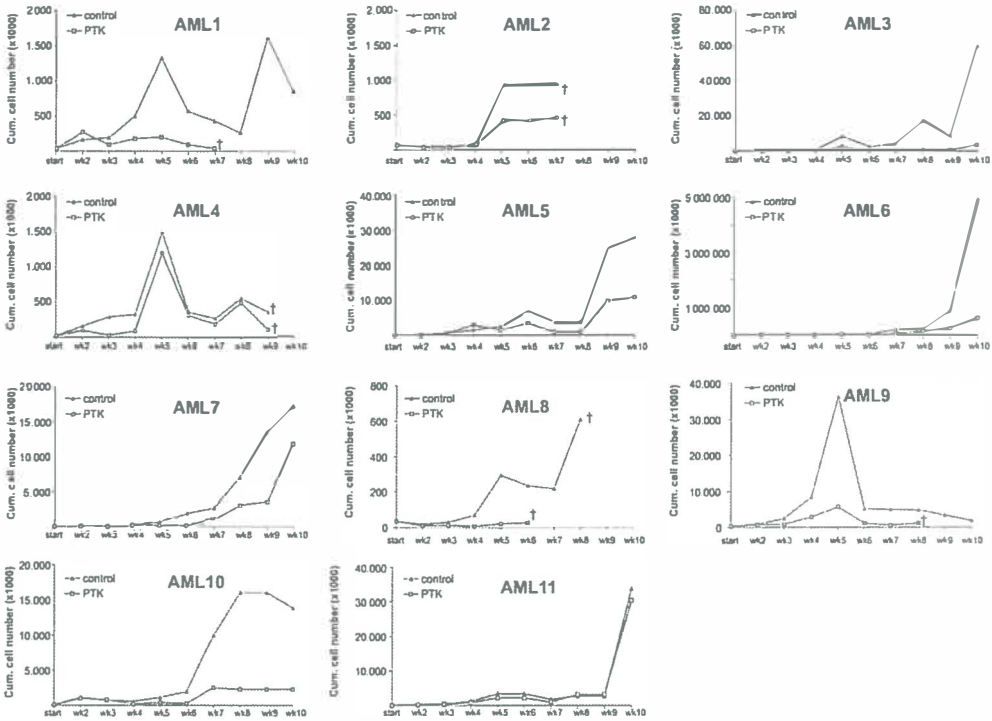
contribute to an impaired outgrowth and a more mature phenotype of these cells. These data indicate that PTK/ZK inhibited leukemic outgrowth and self-renewal potential of CD34+ sorted pediatric AML blasts.

Figure 1. The establishment of long-term cocultures on stromal cells,



(**A**) CD34+ cells of 13 pediatric AML samples were cultured on MS5 and the expansion was observed by counting the non-adherent cells weekly. At week 5, suspension and adherent cells were harvested and replated onto new stroma, indicated by the arrow. Sustained growth up to 10 weeks was seen in 9 out of 13 samples. †: stop leukemic expansion. (**B**) Culture on MS5 stroma showed phase-dark L-CAs underneath the stroma as well as leukemic cells in suspension (bright cells) (AML6 at week 2). L-CAs are encircled. Magnification 400x.

Figure 2. The effect of PTK/ZK on the outgrowth of pediatric LICs.



Growth curves of eleven AMLs are shown. At week 5, the leukemic cells both in suspension and adherent were harvested and replated on new MS5 to initiate secondary cocultures. †: stop leukemic expansion.

Outgrowth and self-renewal of pediatric LICs not affected by addition of VEGFA or treatment with Bevacizumab

Previously it was shown that VEGFA levels at diagnosis are an independent prognostic factor for relapse free survival in (pediatric) AML. We, therefore, specifically determined whether VEGFA has a key role in the effect induced by PTK/ZK. VEGFA mRNA could be detected in all tested patient samples (n=11, Supplementary Fig. S2A). We investigated the effect of VEGFA signaling on the outgrowth of pediatric LICs by addition of VEGFA or treatment with Bevacizumab. To obtain a constant supply of VEGFA in the long-term cocultures, MS5 stromal cells were transduced with a retroviral vector containing VEGFA165. A 30-fold upregulation of VEGFA165 mRNA was achieved in MS5-VEGFA cells compared with MS5-control cells (Supplementary Fig. S2B). Protein VEGFA levels at week 10 were measured and showed that VEGFA production was sustained during the culture (n=2, Supplementary Fig. S2C). In addition, functionality of the protein was confirmed (Supplementary Fig. S2D). Sorted CD34+ cells of six pediatric AML patients were plated onto transduced MS5 stromal cells. No significant effect was seen on the growth of the pediatric LICs when exposed to stroma derived VEGFA compared with culture on MS5-control cells (Supplementary Fig. S2E; n=6, median value). Interestingly, PTK/ZK still remained its effect

when cultured in an environment with VEGF overexpression (by MS5-VEGFA cells). The median value of 6 samples cultured on MS5-VEGFA cells showed a significant ($P<0.05$) inhibited expansion throughout week 2-6 and 8-9 when treated with PTK/ZK compared with control (data not shown). In order to investigate whether selective targeting of the VEGFA-signaling would affect the leukemic outgrowth, we cultured LICs of six pediatric patients with Bevacizumab, a monoclonal antibody to VEGFA. Treatment with Bevacizumab did not result in a significant reduction of leukemic expansion after 10 weeks of culture (Supplementary Fig. S2F n=6, median value). Together, these data suggest that the effect of the tyrosine kinase inhibitor PTK/ZK is not mainly dependent on blocking exogenous VEGFA signaling. Also no response was seen when cultured with a specific antibody for VEGFR2 or VEGFR3, ascribing the results (data not shown).

PTK/ZK exerts its effect via a reduced phosphorylation of downstream targets

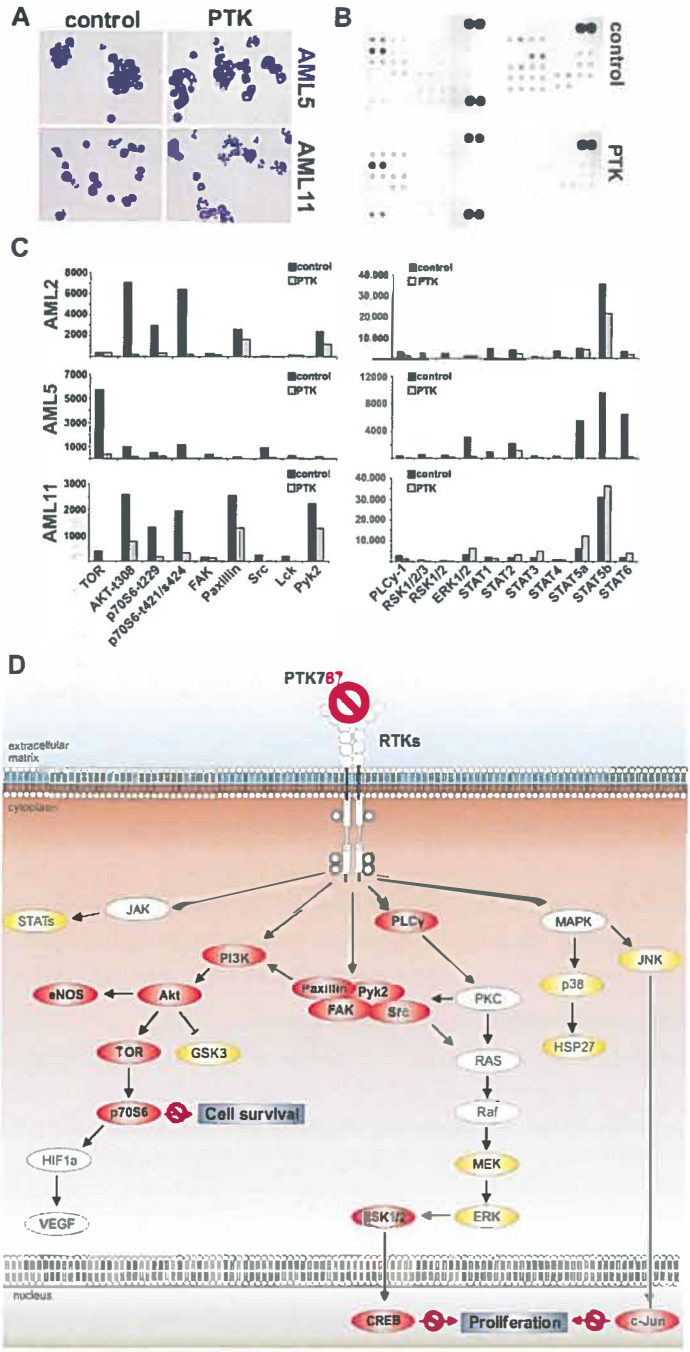
To investigate the mechanisms by which PTK/ZK can inhibit long-term expansion of AML cells we studied the expression of the RTKs inhibited by PTK/ZK. Data on mRNA level showed that expression of most of these RTKs was present in the AML cells although in different expression profiles (Fig. 4A). FACS analysis of the CD34+ sorted AML cells at start of the experiment demonstrated that VEGFR2 expressing cells could be detected in 11 of the 13 AML samples, ranging from 3% to 94% VEGFR2 positive cells (Table 2, for 2 patients representative FACS data are shown in Fig. 4B). FACS results for VEGFR1, VEGFR3, c-KIT, cFMS and PDGFRß are shown in Table 2. Interestingly, PTK/ZK responsiveness was not found to be correlated with the expression of one particular receptor (Table 2).

Table 2. Protein expression of the tyrosine kinase receptors and response of the AML samples to PTK/ZK.

	% growth of control		FACS at the start					
Patient ID	wk5	wk10	VEGFR2	VEGFR1	VEGFR3	PDGFRb	c-KIT	c-FMS
AML1	15	0	9	x	ND	x	ND	x
AML2	45	† (wk 7)	33	x	ND	34	ND	ND
AML3	29	7	42	x	ND	x	3	x
AML4	80	† (wk 9)	4	x	ND	x	ND	x
AML5	62	40	3	x	ND	ND	ND	10
AML6	48	12	94	x	ND	19	ND	24
AML7	4	69	42	x	ND	34	ND	ND
AML8	9	† (wk 8)	3	5	ND	x	ND	x
AML9	15	0	83	5	ND	x	ND	x
AML10	38	17	ND	x	ND	ND	ND	x
AML11	66	90	ND	x	ND	ND	ND	x
AML12	x	x	30	4	ND	x	5	x
AML13	x	x	54	ND	ND	x	ND	x

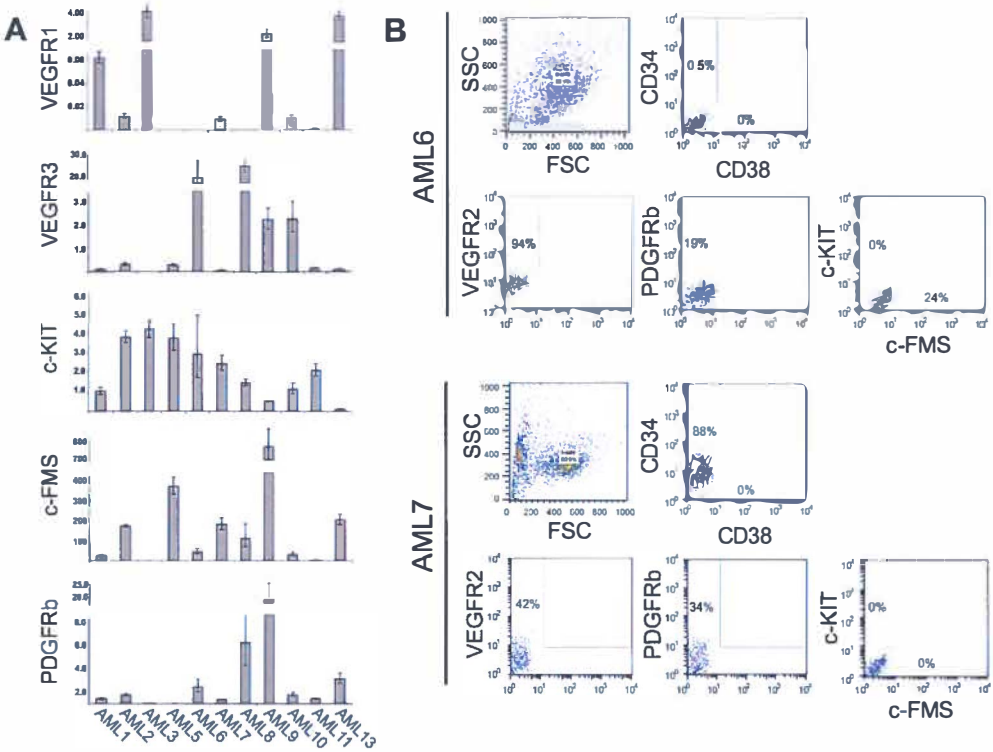
Percentage of CD34+ cells that express protein levels of the tyrosine kinase receptors VEGFR1, VEGFR2, VEGFR3, c-KIT, c-FMS and PDGFRß at the start of the cultures is shown. The response to PTK/ZK is shown, as compared with the growth of control group (% growth of control). † stop leukemic expansion; x Not Available; ND Not Detectable.

Figure 3. The effect of PTK/ZK on phosphorylation of downstream targets in pediatric LICs.



(**A**) Morphological analysis of suspension cells of AML5 and AML11 was performed by MGG staining of cytospins. (**B**) Representative images of Proteome Profiler Human Phospho-Kinase Array on sorted CD34+ cells treated with PTK/ZK or control (AML11). (**C**) Phosphorylation signal intensity of intracellular kinases from three pediatric AML samples treated with or without PTK/ZK, applied to the Proteome Profiler Human Phospho-Kinase Array. (**D**) Figure summarizing the affected downstream targets of PTK/ZK, identified by Proteome Profiler Human Phospho-Kinase Array. Treatment with PTK/ZK downregulated kinases within the PI3K/Akt signaling pathway (PI3K, Akt, TOR, p70S6), and kinases involved in focal adhesion kinase pathway proteins (FAK, Paxillin) and Src kinase pathway proteins (Src, PYK2), resulting in a decreased cell survival. Phosphorylation of the kinases PLCγ, RKS1/2, and CREB involved in the Ras/Raf/MEK/ERK pathway were decreased after addition of PTK/ZK, whereas MEK and ERK were differentially phosphorylated. Phosphorylation of kinases that belong to the MAPK-pathway (p38, HSP27, JNK) and STAT-pathway was differentially effected upon PTK/ZK. A decreased phosphorylation of c-Jun and CREB should result in reduced proliferation. The colors represent lower expressed (red), differentially expressed (yellow) kinases upon treatment with PTK/ZK; white kinases are not measured on the array; → stimulatory effect; ⊣ inhibitory effect.

Figure 4. Protein and mRNA expression of the tyrosine kinase receptors.



(**A**) Relative mRNA expression of VEGFR1, VEGFR2, VEGFR3, c-KIT, c-FMS and PDGFRß for 11 AML samples is shown. (**B**) Percentage cells positive for protein expression of the tyrosine kinase receptors VEGFR1, VEGFR2, VEGFR3, c-KIT, c-FMS and PDGFRß at the start of the cultures was measured by FACS analysis. Data of AML6 and AML7 is shown.

In order to measure downstream effects of the reduced outgrowth of CD34+ pediatric AML cells induced by PTK/ZK, cell lysates of three AML samples treated with or without PTK/ZK were subjected to a Human Phospho-Kinase Array Kit (Fig. 3B). Phosphorylation of PI3K/Akt kinase (AKT, TOR, p70S6K), focal adhesion kinase (FAK, Paxillin) and Src kinase (Src, Lck, PYK2) signaling pathways were reduced upon treatment with PTK/ZK in all three AML samples (Fig. 3C, complete list shown in Supplementary Table S2). Phosphorylation of RAS/RAF/MEK/ERK kinases and STAT kinases was differentially affected upon treatment with PTK/ZK (Fig. 3C). These results indicate that PTK/ZK reduces the expression and/or activation of various oncogenic and angiogenic pathways in CD34+ sorted pediatric AML cells, which may lead to a decrease in cell proliferation (Fig. 3D). In conclusion, treatment of pediatric LICs with PTK/ZK demonstrated various differences in signaling networks, all resulting in cell proliferation and renewal.

Discussion

In this study we showed for the first time that pediatric CD34+ sorted AML cells can be cultured in a long-term leukemic stem/progenitor assay. After replating the cultured AML cells onto new MS5, secondary L-CAs were generated underneath the stroma and maintained self-renewing capacity for up to 10 weeks in 9 of the 13 (69%) pediatric AML samples. Our results are in agreement with data on adult LICs which show that sorted CD34+ cells from adult AML patients can be cultured on a stromal layer [25]. In contrast with results form Gosliga et al., we were able to maintain a long-term culture up to 10 weeks in two samples from good risk patients (AML5 and AML11) although they were characterized by a slower expansion compared with other AML samples.
In our study we cultured sorted CD34- cells of four pediatric AML samples (AML2, AML5, AML6, and AML10) and could not initiate long-term expansion cultures and secondary co-cultures, similar to data from van Gosliga et al (data not shown)[25]. Together, our results are in agreement with data on LTC-IC cultures of adult AML samples and show that the growth kinetics of pediatric AML samples overlaps with the growth features of adult AML cells.

The level of VEGFA at time of diagnosis has been shown to be an independent prognostic factor for treatment outcome in (pediatric) AML (e.g. refractory disease or relapse) [15,17,18]. In addition, increased expression of VEGFA correlated with the enhanced angiogenesis found in AML bone marrow biopsies [28,29]. It had been suggested that LICs reside within 'stem cell niches', consisting among others of a vascular niche formed by sinusoidal endothelium lining blood vessels [4,7,30,31]. Moreover, these bone marrow niches might secrete growth factors including VEGFA [9-11]. Since PTK/ZK was initially designed to block the VEGF/VEGFR signaling pathway with its main effect upon VEGFR2 inhibition, we specifically determined whether VEGFA has a key role in the effect induced by PTK/ZK. No effect was seen on the growth of pediatric samples when exposed to stroma derived VEGFA or cultured in the presence of Bevacizumab, independent on the mRNA VEGFA expression of the AMLs. Together, VEGFA alone does not significantly influence the growth of sorted CD34+ pediatric AML cells. Therefore, VEGF signaling interference does not seem to be the main or only target for the inhibitory effects of PTK/ZK on LICs.

Activation of multiple signal transduction pathways, such as the Ras/Raf/MAPK/ERK, PI3K/Akt/mTOR and Jak/STAT pathways, has a progressively worse adverse effect on AML outcome [32-35]. These downstream intracellular signaling pathways, functioning as an interrelating network, are activated by phosphorylation of RTKs. Our study showed that addition of PTK/ZK reduces the outgrowth of pediatric LICs, whereas a more specified anti-VEGFA antibody has no effect on leukemic outgrowth. Further analysis showed that treatment with PTK/ZK resulted in reduced activation of PI3K/Akt signaling pathway in all tested samples. We hypothesize that PTK/ZK exerts its effect by blockade of multiple RTKs with a decrease in the network of signal transduction pathways, and therefore blockade of only VEGFA is not sufficient to induce a reduced leukemic expansion. These results are in line with new hypotheses that targeting multiple kinase pathways will be more beneficial for AML patients than interfering with single growth factors or RTKs [34].

In conclusion, we demonstrated for the first time that pediatric CD34+ LIC enriched AML cells expand and self-renew in long-term culture assays, and that addition of PTK/ZK to these cultures impairs these processes likely via targeting multiple downstream pathways. Taken together, our work suggests that PTK/ZK might be an effective approach in eradicating the primitive leukemic cell and could be a promising approach for improvement of AML (relapse) therapy.

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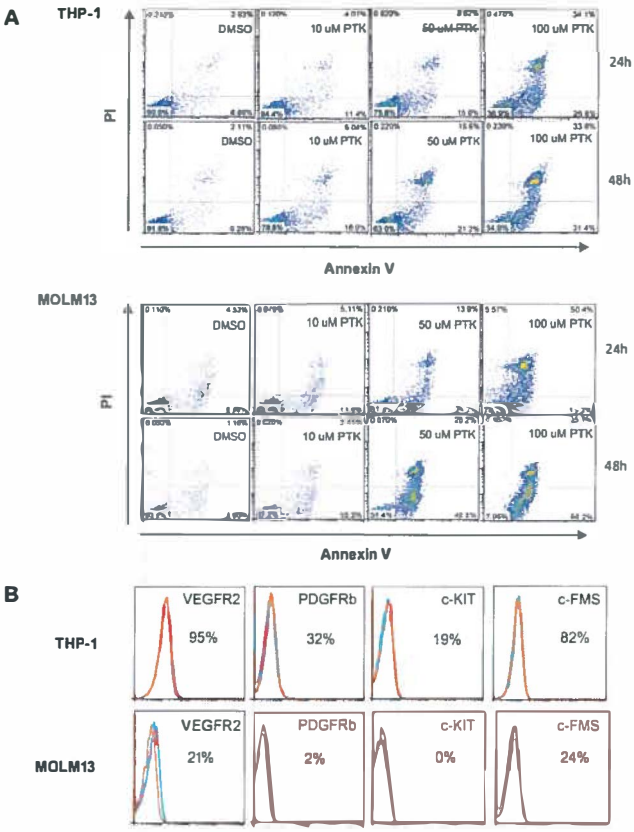
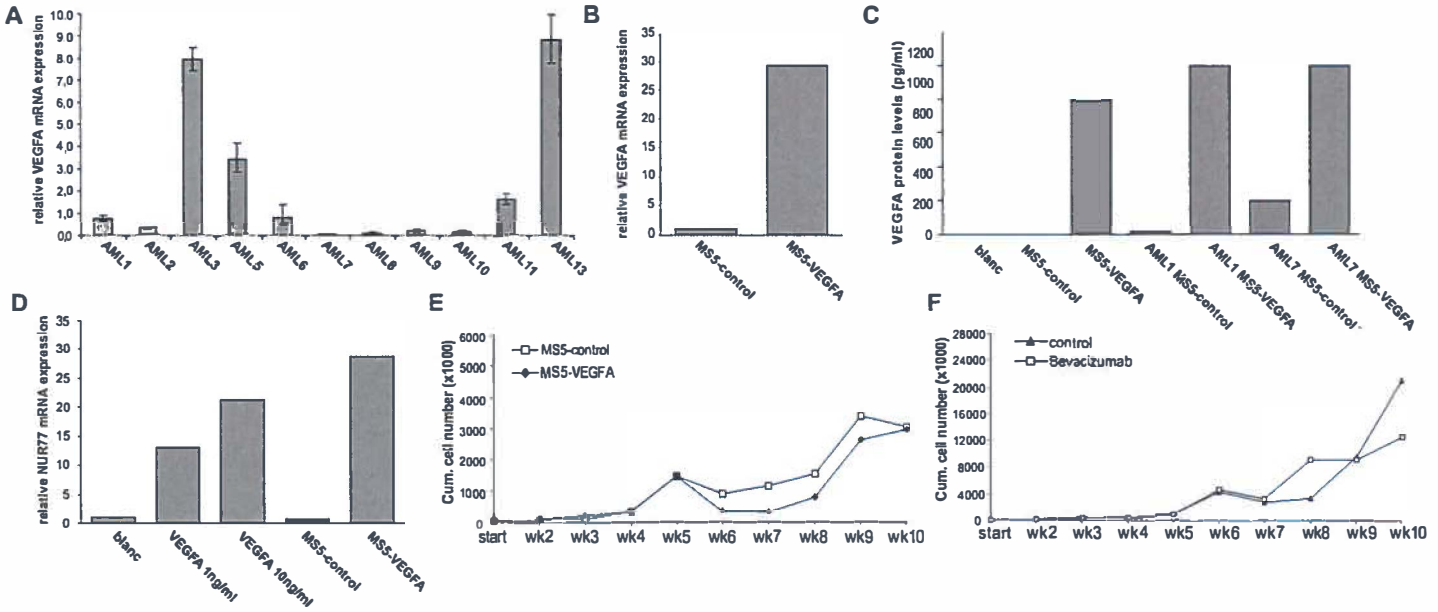
Supplementary data

Supplementary Figure S1. Effect of PTK787/ZK 222584 on AML cell lines THP-1 and MOLM13.



(**A**) A time- and dose-dependent assay for PTK/ZK and control (DMSO) determined by Annexin/PI staining, measured by FACS analysis. Early apoptotic cells are Annexin-positive and PI negative, whereas cells that are in late apoptosis or already dead are both Annexin and PI positive. As shown for both cell lines, an induction of apoptosis was dose-dependent. (**B**) FACS analysis of the tyrosine kinase receptors VEGFR2, c-KIT, c-FMS and PDGFRß. Percentage cells positive for protein expression was measured after 24 hours of culture for both culture conditions. Expression of VEGFR3 was not detectable (not shown). No difference was seen on receptor expression when cultured with PTK/ZK compared with control cultures.

Supplementary Figure S2. VEGFA165 overexpression in MS5 cells.



(**A**) Relative mRNA expression of VEGFA for 11 AML samples is shown. (**B**) Relative mRNA expression of VEGFA165 in MS5-control compared with MS5-VEGFA. A 27-fold upregulation was found. (**C**) ELISA confirmed secreted VEGFA protein levels in the supernatant of the MS5-VEGFA cells (751 pg/ml), whereas VEGFA was not detectable in supernatant of the MS5-control cells. ELISA of supernatant of AML samples cultured for 10 weeks showed that VEGFA protein production was sustained during culture. (**D**) Recombinant VEGFA induces expression of NUR77 in ECs in a dose dependent way. Supernatant of transduced MS5 cells contained functional VEGFA, indicated by the expression of NUR77. MS5-VEGFA showed a higher expression of NURR77 than MS5-control, demonstrating that VEGFA165 cells produce more functional VEGFA. Similar graphs were seen when investigating the expression of EGR3 and NOR1. (**E**) Median growth curve of sorted CD34+ cells of 6 pediatric patients cultured on MS5-control or MS5-VEGFA165. No significant difference in expansion was seen between both conditions (paired Wilcoxon signed rank test, week 2-10, P>.05). (**F**) Median growth curve of sorted CD34+ cells of 6 pediatric patients cultured with or without addition of Bevacizumab. No significant difference in expansion was seen between both conditions (paired Wilcoxon signed rank test, week 2-10, P>.05).

Supplementary Table S1. Specific primers for RT-PCR.

Supplementary Table S2. Phosphorylation of downstream intracellular kinases.

Cell lysates of three AML samples treated with or without PTK/ZK were subjected to a Human Phospho-Kinase Array Kit. The list displays the phosphorylation data of the downstream intracellular kinases.

Chapter 5

Stromal interaction essential for Vascular Endothelial Growth Factor A induced tumour growth via Transforming Growth Factor ß signalling

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Abstract

Background: High Vascular Endothelial Growth Factor (VEGFA) levels at time of diagnosis confer a worse prognosis to multiple malignancies. Our aim was to investigate the role of VEGFA in promoting tumour growth through interaction with its environment.

Methods: HL-60 cells were transduced with VEGFA165 or control vector using retroviral constructs. VEGFA165 cells (n=7) or control cells (n=7) were subcutaneously injected into NOD/SCID mice. Immunohistochemistry of markers for angiogenesis (CD31) and cell proliferation (Ki67) and gene expression profiling of tumours was performed. Paracrine effects were investigated by mouse-specific cytokine arrays.

Results: *In vivo* we observed a twofold increase in tumour weight when VEGFA165 was overexpressed (P=0.001), combined with increased angiogenesis (P=0.002) and enhanced tumour cell proliferation (P=0.001). Gene expression profiling revealed human genes involved in TGF-ß signalling differentially expressed between both tumour groups, i.e. TGFBR2 and SMAD5 were lower expressed whereas the inhibitory SMAD7 was higher expressed with VEGFA165. An increased expression of mouse-derived cytokines IFNG and IL7 was found in VEGFA165 tumours, both described to induce SMAD7 expression.

Conclusion: These results suggest a role for VEGFA-driven tumour growth by TGF-ß signalling inhibition via paracrine mechanisms *in vivo*, and underscore the importance of stromal interaction in the VEGFA-induced phenotype.

Introduction

The level of Vascular Endothelial Growth Factor (VEGFA) at time of diagnosis is an independent prognostic factor for treatment outcome in multiple malignancies, including (paediatric) acute myeloid leukaemia (AML) [1,2]. As a result, targeting VEGFA has been subject of extensive research during the last decades.

In general, tumour growth is supported by angiogenesis, the formation of new blood vessels. An increase in microvessel density (MVD) is seen in many malignancies. In AML, enhanced bone marrow angiogenesis at diagnosis was shown, recovering to normal levels when CR was achieved [3,4]. Moreover, a correlation was found between the degree of VEGFA expression and the increase in bone marrow vascularization at AML presentation [3].

Co-expression of VEGFA and its tyrosine kinase receptors VEGFR1 and VEGFR2 has been reported previously in AML. Upon binding of its ligand, both receptors can activate several downstream pathways such as PI3-kinase and MAPK signalling, inducing cell proliferation and cell survival [5-7]. Apart from autocrine effects, secreted VEGFA stimulates endothelial and stromal cells that may drive leukemic cell proliferation via paracrine effects [8,9].

Although a role for VEGFA in malignant progression was implicated, the exact mechanism by how increased VEGFA levels influence tumour growth is still not completely understood. In this study a model was generated to investigate the effect of VEGFA in tumour progression. Using a human AML cell line transduced with VEGFA165 or empty vector (negative control), we were able to show that *in vitro* overexpression of VEGFA165 did not influence cell growth patterns and drug resistance, whereas *in vivo* VEGFA165 overexpression enhanced tumour outgrowth and induced the expression of host-derived cytokines. Our study suggests that VEGFA165 promoted tumour growth was mediated by a paracrine induced inhibition of the TGF-β signalling pathway, and underlines the importance of targeting the tumour microenvironment.

Materials and methods

Cell line culture and transduction

The human acute myeloid leukaemia cell line HL-60 was obtained from the ATCC (Manassas, VA) in 2006. Cells have last been tested and authenticated by flow-cytometry analysis in 2009. HL-60 cells were cultured in RPMI-1640 medium supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS, Hyclone, Logan, Utah, USA). Retroviral supernatants were generated by cotransfection of 2 μg reporter constructs pMSCV-iGFP-VEGFA165 or pMSCV-iGFP (empty vector, negative control) and 2 μg packaging plasmid pCLampho into 293T cells using FuGENE HD transfection reagent (Roche, Almere, the Netherlands). 5×10^4 HL-60 cells were incubated with retroviral supernatants which were filtered through 0.45-μm pore size syringe-mounted filters. Incubation was supplemented with 8 μg/ml polybrene. This

procedure was repeated for two consecutive days after which stably transduced cells were expanded. Transduction efficiency was measured by FACS analysis which demonstrated an efficiency of 25% for cells transduced with the empty vector and 10% for cells transduced with VEGFA165. Cells were sorted on a MoFlo (DAKO Cytomation, AS, Glostrup, Denmark); after sort the percentage transduced cells was 90% for the empty vector (control cells) and 67% for the VEGFA165 vector (VEGFA165 cells). Cells were cultured overnight under described serum-conditions before use.

Animal study

NOD/SCID mice (kindly provided by dr. L.D. Shultz, The Jackson Laboratory, Bar Harbor, ME) were bred and maintained at the Central Animal Facility, University of Groningen, Groningen, the Netherlands. Animals were kept under laminar flow conditions during the experiment. Our model was generated by subcutaneously injecting $10x10^6$ VEGFA165 cells (VEGFA165 tumour) or control cells (control tumour) into the right flank of sublethally irradiated (2 Gy) 6–8-week-old NOD/SCID mice (n=14). Mice were monitored for 15 days, during which tumour growth was assessed periodically. Tumour volumes were determined by external measurement according to the equation $[V=L\times W^2]\times 0.5$, where V=volume, L=length and W=width [10]. Mice were sacrificed using cervical dislocation at day 15 and tumours were harvested. Tumours were split and either snap-frozen or fixed in formalin and embedded in paraffin for immunohistochemistry or other analyses. All procedures involving animals were performed in accordance with local ethical animal laws and policies.

RNA extraction and real-time PCR

Total RNA from frozen tumour material was extracted with NucleoSpin RNA II kit according to manufacturer's protocol (Macherey-Nagel, Duren, Germany). cDNA was prepared at 37°C for at least one hour in 20 µl reaction mixture containing 2 µg of total RNA, random hexamers (Pfizer, Capelle a/d IJssel, The Netherlands), 5x first strand buffer, RNAsin and reverse transcriptase (Gibco BRL, Grand Island, NY, USA). Real-time PCR was performed using iQ SYBR green supermix (Bio-Rad, Hercules, CA, USA). All PCR reactions and data analysis were performed on the iCycler iQ Real-Time Detection System (Bio-Rad). Expression of genes of interest was standardized for expression of hRPL22 or mβ-actin (Arbitrary Units, AU). Human-specific (h) and mouse specific (m) primers for the RT-PCR are listed in Online Supplementary Table S1. The primers were checked in NCBI-blasts and did not occur in mouse genome or transcriptome. In addition, no PCR product could be detected in mouse tissue with these primers.

ELISA and functional assay of VEGFA

Secretion of VEGFA was detected in supernatant using commercially available ELISAs (Quantikine immunoassays, R&D systems, Abingdon, United Kingdom) following manufacture's instructions. The functionality of secreted VEGFA from transduced cells was detected by adding its supernatant to endothelial cells and quantify expression of VEGFA specific genes EGR3, NUR77 and NOR1 in endothelial cells with real-time PCR, described in detail by Lui et al [11].

Flow cytometry analysis

For BrdU incorporation, a measure of DNA-synthesis, $0.5x10^6$ cells were incubated for 2, 4 or 8 hours with 0% or 10% FCS in presence of 1 µM Demecolcine (Sigma) and 10 µM BrdU (BD, Alphen a/d Rijn, the Netherlands), and prepared according to the BdrU-assay protocol following manufacturer's instructions (BD); cells were neutralized for acids in 0.5M EDTA, secondary antibody: PE-conjugated anti-mouse (DAKO). Measurements with FACScalibur LSR-II (BD), data were analyzed using FlowJo.

Cellular drug resistance measurement using total cell kill assay

A total cell kill assay was performed on VEGFA165 cells and control cells using Amsacrine (0,05–2 µg/ml) in different concentrations in quadruplicate following former publications [12]. Optical Density (OD) in the total cell kill assay is linearly related to the number of viable cells.

Microarray analysis

RNA from 14 tumour samples was analyzed using Affymetrix Human Genome U133 Plus 2.0 GeneChip (Affymetrix, Santa Clara, CA, USA). RNA quality control, cDNA labelling, microarray hybridization, scanning, data extraction and data normalization were performed by ServiceXS, Leiden, the Netherlands. Differentially expressed genes were identified for tumours using multivariate permutation test in BRB ArrayTools. A total of 1000 permutations were completed to identify the list of probe sets. Differentially expressed probe sets were identified using a two-sample T-test, threshold $P<0.001$ and False Discovery Rate (FDR) of <0.25 was used. Gene Ontology (GO) categories and KEGG pathways were determined using DAVID (Database for Annotation, Visualization, and Integrated Discovery). Probe sets with a significance level of 0.001 and FDR <0.25 were used for GO analysis. Gene set expression comparisons were performed with univariate two sample T-test at a significance level of $P<.05$, followed by 200 permutations. Affymetrix array results were validated using QRT-PCR with human-specific primers for eleven genes of interest (Supplementary Figure S1).

Immunohistochemical analysis for vessel density and proliferation

Frozen tumour samples were cut into 4-µm sections and studied for vessel density by staining with CD31 [platelet endothelial cell adhesion molecule (PECAM)-1] as well as for proliferation by staining with Ki67. Sections were blocked for endogene peroxidase with 0.25% H_2O_2 and incubated with mouse-specific CD31 (BD). Subsequently, sections were incubated with secondary antibody (swine) anti-rat biotin (DAKO), amplified with (biotin)streptavidin ABComplex/HRPO (DAKO) and detected by 3-amino-9-ethylcarbazole (Sigma). After that slides are coloured with hematoxillin. Negative controls were produced using non-specific IgG as primary antibody. Sections for Ki67 were treated similar except for the antibodies: primary monoclonal mouse anti-human Ki67 antibody (DAKO) and secondary antibody (rabbit) anti-mouse biotin (DAKO). Vessel density was assessed using light microscopy at 50x magnification in areas of the slide containing the highest numbers of microvessels representing most intense microvasculature (hotspots). After the hotspots were identified, total number of vessels per selected image was counted at 400x magnification. At least four hotspots were counted for each

section. Proliferation was evaluated by percentage of Ki67 positive tumour cells in four hotspot areas (selected at 50x magnification): the selected image was divided into four areas, and the percentage Ki67 positive tumour cells was estimated for all four areas at 400x magnification. Mean of the four estimates was considered the count of that hotspot. Stainings were evaluated by two investigators who had no knowledge of tumour characteristics. Variability between investigators for vessel count was rho=0.993 and for proliferation count was rho=0.996. Mean of the two independent counts was considered to be final measurement for each counting field and hotspot.

Cytokine array

In VEGFA165 (n=4) and control (n=4) tumours 40 mouse-specific cytokine levels were determined by using proteome profiler mouse cytokine array panel A kit (R&D Systems) according to manufacture's instructions (400 µg protein of each sample was added). Spot densities were quantified with Scanalyze software (http://rana.lbl.gov/EisenSoftware.htm) and exported to Microsoft Excel. Spot densities were corrected for individual background to diminish interarray variances. To assess the mouse specificity of the cytokine array, protein extracts of the human HL-60 cell line and of the mouse MS5 cell line were applied to the cytokine array. As expected, a strong signal was found for the MS5 mouse cells, and no signal could be detected for the HL-60 control cells (Supplementary Figure S2).

Statistical analysis

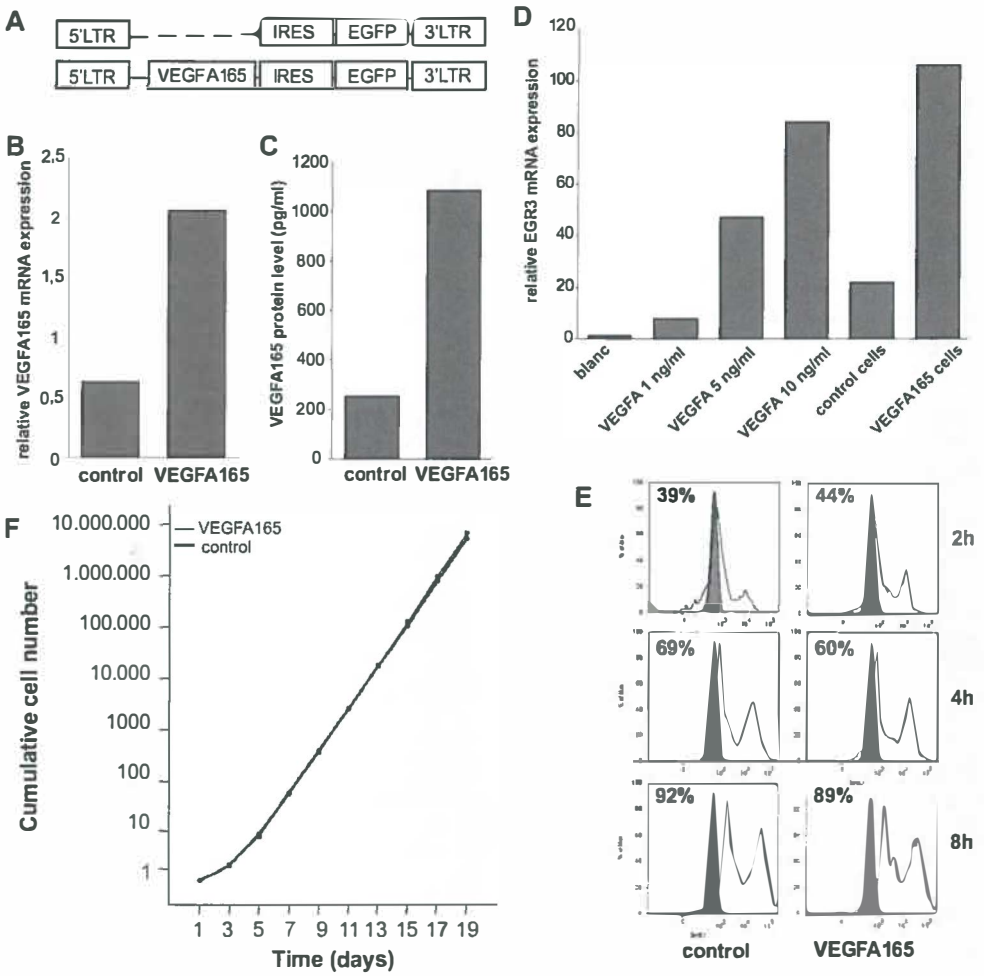
Wilcoxon signed rank test was used to compare growth of both cell lines. Tumour volumes between VEGFA165 tumours and control tumours were determined using the Student's t-test. Differences between tumour volumes at indicated time points were calculated using the Student's paired t-test. Correlation between expression of VEGFA165, tumour volume, tumour weight, percentage cell proliferation and MVD were calculated using Spearman's correlation. Mann-Whitney U test was used for comparison of vessel and proliferation counts, QRT-PCR as well as mouse-specific cytokines differentially expressed within VEGFA165 tumours and control tumours. $P<0.05$ was considered significant.

Results

Functional VEGFA165 overexpression in HL-60 cells

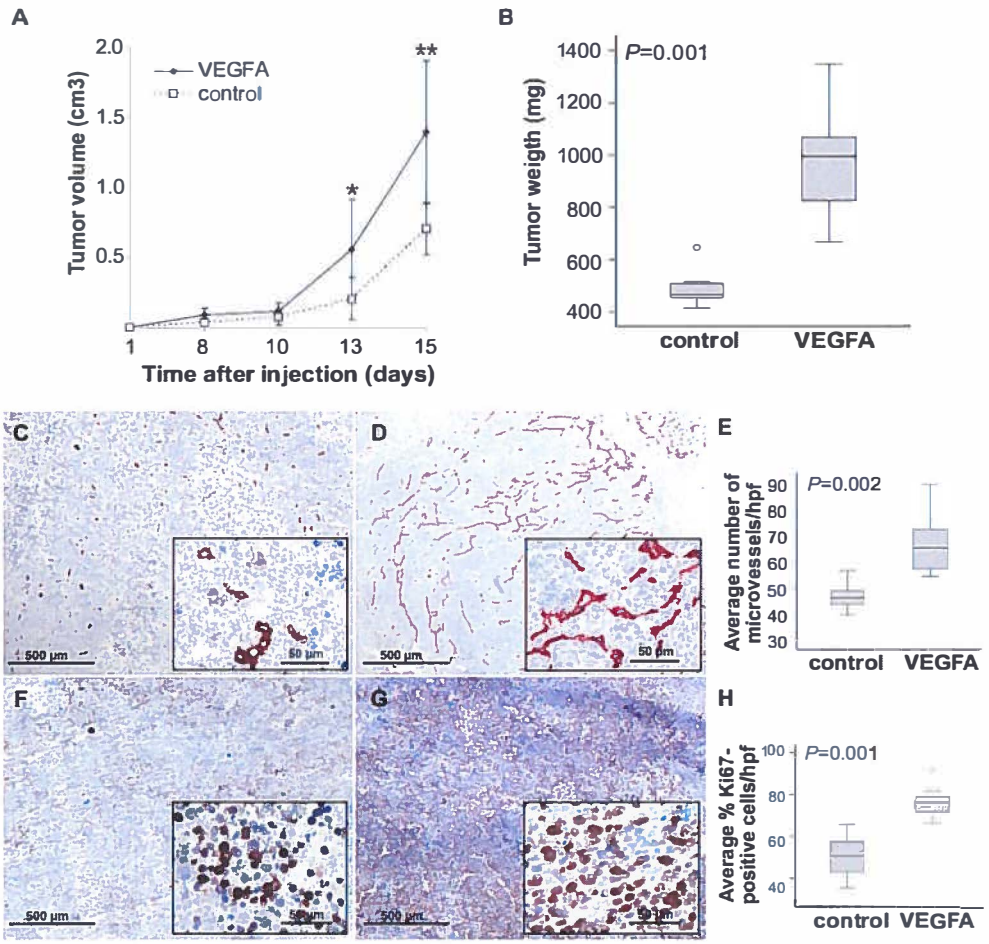
We transduced HL-60 cells with VEGFA165 (retroviral vectors schematically depicted in Figure 1A). VEGFA165 mRNA expression and secreted protein levels were 3-4 fold upregulated in VEGFA165 cells compared with control cells (Figure 1B-C). To test the functionality of the produced VEGFA165, conditioned medium of VEGFA165 transduced cells or control cells was added to endothelial cells (HUVECs) and the mRNA expression of a VEGFA responsive gene EGR3 was measured. A fivefold upregulation of EGR3 was detected in HUVEC cells after incubation with conditioned medium of VEGFA165 transduced cells compared with conditioned medium of control cells (Figure 1C). FACS analysis showed KDR expression in VEGFA165 transduced cells similar to control cells. *In vitro* cell proliferation and cell growth was similar in both cell lines (Figure 1D-E) and no difference in drug resistance was observed between VEGFA165 cells and control cells (data not shown).

Figure 1. Effect of VEGFA165 overexpression *in vitro*.



(**A**) Schematic representation of the retroviral constructs that were used in this study. (**B**) Relative mRNA expression of VEGFA165 in control cells compared with VEGFA165 cells. (**C**) Protein expression of VEGFA165 in control cells compared with VEGFA165 cells: control cells 254.3 pg/ml, VEGFA165 cells 1085.5 pg/ml. (**D**) Functionality of the produced VEGFA by transduced cells. Recombinant VEGFA induces expression of EGR3 in ECs in a dose dependent way (1 ng/ml: 7.8 AU, 5 ng/ml: 47.3 AU, 10 ng/ml: 84.3 AU). Supernatant of transduced cells contained functional VEGFA, indicated by the expression of EGR3. VEGFA165 cells showed a higher expression of EGR3 than the control cells, demonstrating that VEGFA165 cells produce more functional VEGFA (control cells: 22.0 AU; VEGFA165 cells: 106.5 AU). Similar graphs were seen when investigating the expression of NUR77 and NOR1. (**E**) BdrU incorporation of control cells and VEGFA165 cells culture in serumfree conditions. After 2h, 4h and 8h cells were harvested and the percentage cells that incorporated BrdU was analyzed by flow cytometry. Percentage BrdU incorporating cells: after 2h 39% versus 44%; after 4h 69% versus 60%; after 8h 92% vs 89 %. Background staining in the absence of BrdU was ~1%. Similar graphs were seen when cells were cultured with 10% FCS. (**F**) No significant difference in cell growth of VEGFA165 cells compared with control cells was seen when cells were cultured with 10% FCS (P=0.169, Wilcoxon Signed Rank test).

Figure 2. Effect of VEGFA165 overexpression *in vivo*.



(**A**) Tumour volumes were assessed periodically. At day 13 and day 15 a significant difference was seen. Values expressed are the means ± SEM of 7 xenografts per group. * $P<0.05$, ** $P<0.01$. (**B**) Tumour weight of tumours derived from VEGFA165 cells was significantly increased compared with tumour weight of control tumours at day 15 ($P=.001$). Median weight VEGFA165 tumours: 995 mg (range 670-1344), control tumours: 464 mg (range 413-646). (**C**) CD31 staining of a VEGFA165 tumour and (**D**) a control tumour. (**E**) VEGFA165 tumours showed a significantly ($P=.002$) higher MVD compared with the control tumours. Median MVD control tumours: 46.1 per field (range 39.9–56.5); median MVDVEGFA165 tumours: 64.9 vessels per field (range 54.4–88.6). (**F**) Ki67 staining of a VEGFA165 tumour and (**G**) control tumour. (**H**) A significantly ($P=.001$) higher percentage proliferating cells was found in the VEGFA165 tumours compared with the control tumours. Median percentage proliferating cells control tumours: 50.3% (range 35.4–65.6); median percentage proliferating cells VEGFA165 tumours: 76.3% (range 66.3–91.6). A representative photograph from each group is shown (50x original magnification, with inset 400x magnification).

Overexpression of VEGFA165 results in increased angiogenesis and cell proliferation in a s.c. xenograft mouse model

To investigate the role of VEGFA165 overexpression *in vivo*, we subcutaneously inoculated VEGFA165 cells or control cells in mice. Tumour volume of VEGFA165 tumours increased more rapidly than control tumours, with a significant difference at day 13 and 15 ($P<.05$ and $P<.01$ respectively, Figure 2A). When tumour-bearing mice were sacrificed at day 15 tumour weight of VEGFA165 tumours (median weight 995 mg, range 670-1344) was significantly ($P=0.001$) increased compared with the control tumours (median weight 464 mg, range 413-646) (Figure 2B). Microvessel density (MVD) in VEGFA165 tumours (median MVD 64.9 vessels/hpf, range 54.4-88.6) was significantly ($P=.002$) enhanced compared with control tumours (median MVD 46.1 vessels/hpf, range 39.9–56.5), and a significant ($P=.001$) increase in tumour cell proliferation fraction in VEGFA165 tumours was found (median: 76.3%, range 66.3–91.6) compared with control tumours (median: 50.3%, range 35.4–65.6) (Figure 2C-H). Interestingly, tumour weight was significantly correlated to MVD (rho=0.566, $P=.044$) and percentage proliferating cells (rho=0.722, $P=.004$). In contrast to *in vitro* results, *in vivo* a clear phenotype could be appreciated as increased angiogenesis and tumour cell proliferation was evident.

Distinct gene expression profiles related to VEGFA165 overexpression *in vivo*

To obtain a more detailed understanding of the phenotype within the tumour cells overexpressing VEGFA165, mRNA of VEGFA165 tumours and control tumours was isolated, amplified, and hybridized to Affymetrix human U133 Plus 2.0 GeneChips. Class comparison analysis revealed 761 probe sets to be differentially expressed between the two tumour groups; 242 probe sets were higher expressed in VEGFA165 tumours whereas 519 probe sets were lower expressed in these tumours (complete list shown in Online Supplementary Table S2). As expected, VEGFA was significantly higher expressed in the VEGFA165 tumours compared with control tumours ($P<1.10^{-5}$).

Subsequently, GO analysis of the upregulated probe sets in VEGFA165 tumours revealed enrichment for the process of 'angiogenesis' (GO:0001525, $P=0.03$) in accordance with the immunohistochemical staining for MVD. The GO term 'cell death' (GO: 0008219, $P=0.02$) was enriched in the downregulated probe sets of the VEGFA165 tumours, underscoring the observed increase in tumour growth and cell proliferation (complete list shown in Online Supplementary Table S3).

The list of up- and downregulated genes in VEGFA165 tumours include known genes involved in neoplasia (e.g. S100A8 involved in leukaemia; S100A9 in prostate carcinoma; BCL9, CCND1 and CTNNB1) [13-16]. In addition, the cytokine receptors IL4R and IL7R were found to be higher expressed in VEGFA165 tumours implicating the possibility of paracrine effects.

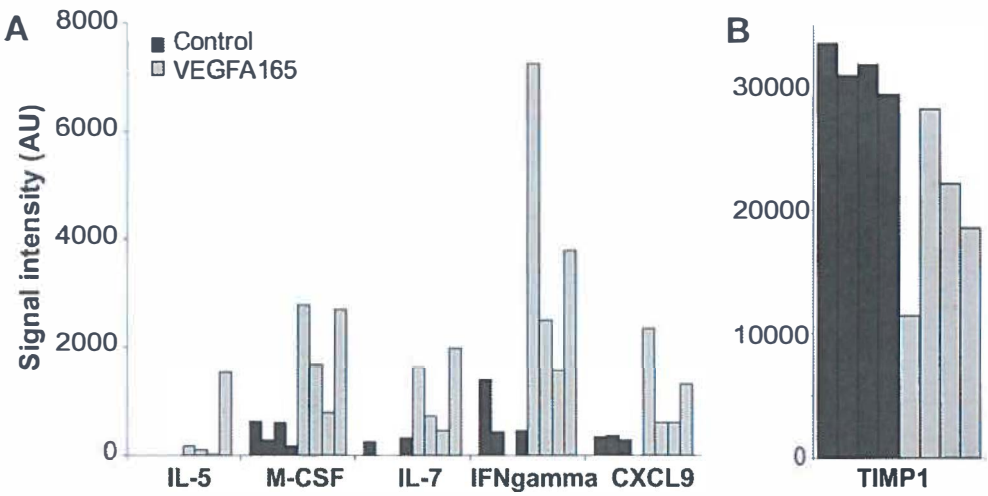
Notable, genes involved in TGF-ß signalling (SOS1, SOS2, SMAD5, LTBP3, TGFBR2, IRF7 and CREBZF) were found to be significantly ($P<0.01$) downregulated in the VEGFA165 tumours. In addition, SMAD7, a negative modulator of TGF-ß signalling, was found to be upregulated in VEGFA165 tumours. Three other genes involved in the TGF-ß signalling pathway were also found to be significantly ($P<0.05$) differentially expressed (SMAD3, IRF7, SMURF2).

To validate these results QRT-PCR with human-specific primers was performed for eleven genes of interest (hVEGFA, hCREBZF, hLTBP3, hIL4R, hIL7R, hSOS1, hSOS2, hSMAD3, hSMAD7, hSMURF2 and hTGFBR2). Differential expression of these eleven genes could be confirmed using QRT-PCR (Supplementary Figure S1). Therefore, we conclude that the genes involved in the TGF-ß signalling pathway are derived from human cells within the tumours.

Paracrine mechanisms play important roles in VEGFA165 tumours

We hypothesized that the *in vivo* growth benefit of high VEGFA165 levels occurs via interaction with its (micro)environment. In the VEGFA165 tumours mouse specific VEGFR2 mRNA was twofold upregulated (P=0.001, Supplementary Figure S1), the main receptor for VEGFA signaling. Using mouse-specific cytokine arrays (tested for mouse specificity, Supplementary Figure S2), we assessed whether the increased tumour growth would be reflected in altered mouse derived cytokine expression. Five mouse-specific cytokines were significantly (P=0.03) higher expressed in VEGFA165 tumours compared with control tumours: interleukin 5 (IL5), interleukin 7 (IL7), chemokine (C-X-C motif) ligand 9 (CXCL9, MIG), colony stimulating factor 1 (macrophage) (MCSF) and interferon gamma (IFNG) (Figure 3A). Tissue metallopeptidase inhibitor 1 (TIMP1) was found to be significantly (P=0.03) lower expressed in VEGFA165 tumours compared with control tumours (Figure 3B). Together these data show that stroma-derived (i.e. mouse-derived) cytokines are regulated in tumours overexpressing VEGFA suggesting that VEGFA exerts its effect on tumour growth via a paracrine loop.

Figure 3. Expression of mouse-specific cytokines.



Mouse specific cytokines significantly (P=0.03) differentially expressed between the VEGFA165 tumours (n=4) and control tumours (n=4), showing 5 cytokines higher expressed (**A**), and one cytokine lower expressed in the VEGFA165 overexpressing tumours (**B**).

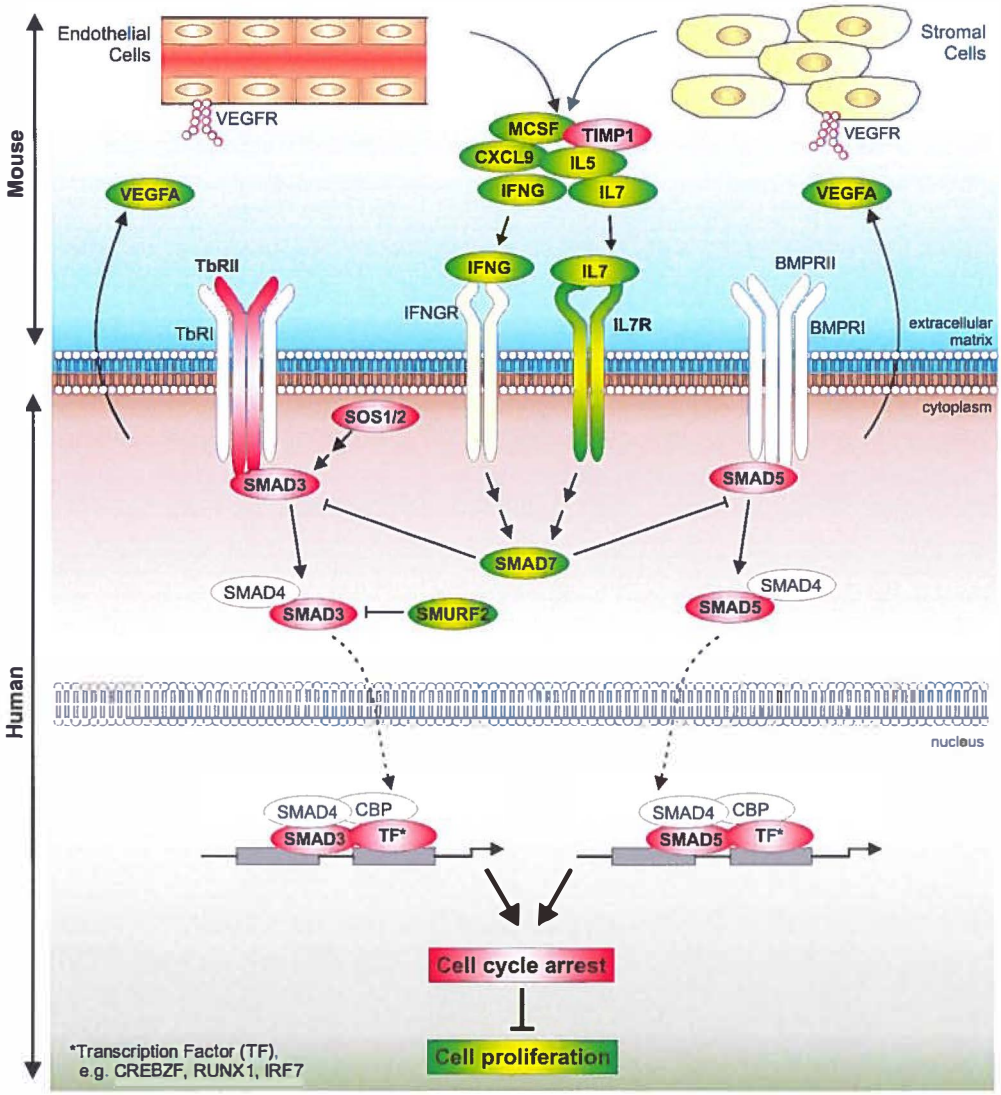
Discussion

In this study we demonstrated that VEGFA165 overexpression significantly enhanced tumour progression *in vivo*, accompanied by a higher percentage proliferating tumour cells and increased angiogenesis. We hypothesize that the enhanced tumour cell proliferation is accompanied by inhibition of the TGF-ß signalling pathway in the (human) tumour cells via various stromal derived mouse cytokines such as IL7 and IFNG (Figure 4).

High VEGFA levels predict a poor prognosis in multiple malignancies, including haematological malignancies. In our study, *in vitro* induction of high VEGFA165 levels did not alter the proliferative status of VEGFA165 cells compared with control cells despite the fact that HL-60 cells express VEGFR2 and the possibility of an autocrine loop has been described [12]. In contrast, overexpression of VEGFA165 *in vivo* resulted in an increased tumour growth and higher levels proliferating cells.

Our data show that tumours overexpressing VEGFA165 were characterized by enhanced vessel outgrowth. An increase in microvessel density (MVD) is seen in many malignancies, including haematological malignancies, with VEGFA as a key player [3,4,17]. Vessels are known for their supply of oxygen and nutrients to the tumour cells, but also for excretion of various endothelial derived growth factors of potential benefit for tumour cells [8,18]. Our results show that endothelial and/or stromal cells within the tumour express higher levels of mouse-specific cytokines (MCSF, IFNG, IL5, IL7 and CXCL9) in VEGFA165 tumours compared with control tumours (Figure 4). From the literature it is known that exposure of human endothelial cells to VEGFA resulted in an increased production of cytokines, including MCSF [5,8,18]. These results suggest that the effect of VEGFA165 is stromal dependent.
New is that within the human tumour cells genes involved in the TGF-ß signalling pathway were found to be differentially expressed between VEGFA165 tumours and control tumours. The TGF-ß signalling pathway is a negative regulator of cell cycle progression, resulting in a cell cycle arrest via transcription of TGF-ß-responsive genes. TGFBR2, SMAD3, SMAD5, SOS1, and SOS2 were found to be lower expressed in VEGFA165 tumours compared with control tumours. In addition SMAD7 was found to be higher expressed in VEGFA165 tumours compared with control tumours. SMAD7 inhibits TGF-ß signalling by preventing the activation of other SMADs [19]. These results suggest that the block in cell cycle progression is decreased via downregulation of TGF-ß-responsive genes, resulting in cell proliferation. This is in line with our observation that VEGFA165 tumours showed an increased fraction of proliferating cells.
Since no proliferative advantage was seen for the VEGFA165 cells in vitro we hypothesized that the TGF-ß signalling pathway is a result of the interaction with environment. Gene expression analysis revealed that nearly all of the genes involved in the TGF-ß signalling pathway were not differentially expressed between the VEGFA165 HL-60 cell lines and control HL-60 cell lines. Thus, our results are in concordance with the knowledge that stromal support is essential in tumour growth in vivo [20].

Figure 4. Proposed mechanism for VEGFA165 induced tumour growth.



VEGFA production from the tumour cells results in an increased fraction of host-derived stromal and endothelial cells. These host-derived supportive cells produce a variety of cytokines that act on the tumour cells. As a result, genes involved in the TGF-ß signalling pathway are differentially expressed, inducing the inhibition of TGF-ß signalling. This, in turn, results in decreased cell cycle arrest, which may explain the increased growth of VEGFA overexpression in tumour. The colours represent higher expressed (green) and lower expressed (red) genes or cytokines within the VEGFA165 tumours; → stimulatory effect; →→ indirect stimulatory effect; —| inhibitory effect; - - > translocation.

Alterations in the TGF-ß signalling pathway are found in human malignancies, including haematological malignancies [21,22]. Proposed mechanisms for the reduced TGF-ß signalling include a decrease in TGFBR2 expression or an increase in SMAD7 expression [19,23]. In our study, tumours overexpressing VEGFA165 showed lower expression of TGFBR2 and higher expression of SMAD7, suggesting a resistance to the TGF-ß induced cell cycle arrest. The expression of SMAD7 can be regulated by various genes and cytokines, e.g. IFNG and IL7 [24,25]. Our data revealed that, in response to VEGFA expression by the tumour cells, these host-derived cytokines were upregulated. In addition, our microarray data showed that (human) IL7 receptor (IL7R) was upregulated in the VEGFA165 tumours. Together, these data suggest that VEGFA165 induces the expression of host-derived growth factors and cytokines, which in turn induces inhibition of the TGF-ß signalling pathway (Figure 4).

In conclusion, we show that VEGFA165 significantly enhances tumour growth and tumour angiogenesis. We demonstrated that tumour derived VEGFA resulted in enhanced tumour cell proliferation possibly by a paracrine inhibition of TGF-ß signalling within the tumour. These results indicate that the microenvironment is essential for VEGFA induced tumour growth. Combining conventional therapeutic strategies with drugs targeting the tumour environment may be of benefit for tumour treatment.

Acknowledgements

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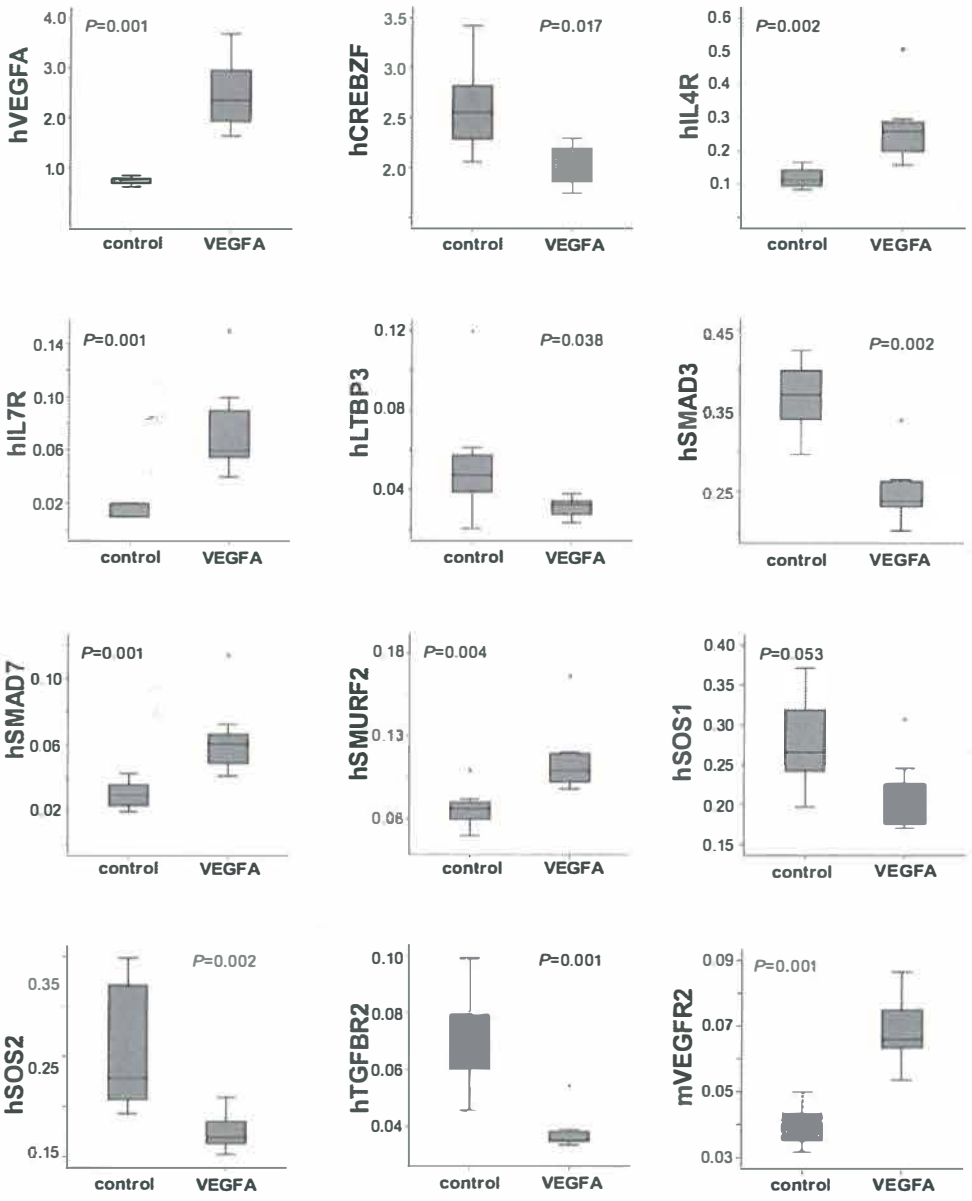
Supplementary Data:

Supplementary Figure S1. Genes differentially expressed between the VEGFA165 tumours (n=7) and control tumours (n=7).



Relative mRNA expression of twelve genes of interest in control tumour cells compared with VEGFA165 tumour cells.

Supplementary Figure S2. Verification of mouse-specific cytokine array.

Mouse specificity was verified by adding protein extracts of mouse cell line MS5 or human cell line HL-60 to the mouse-specific cytokine array. Positive controls are shown on both blots. A strong signal was found for the MS5 mouse cells, and no signal could be detected for the HL-60 control cells.

Supplementary Table S1. Human-specific (h) and mouse-specific (m) primers for RT-PCR.

Supplementary Table S2. Probes differentially expressed between the VEGFA165 tumours (n=7) and control tumours (n=7) P=0.05.

Supplementary Table S3. Enrichment of GO terms among differentially expressed probes between the VEGFA165 tumours (n=7) and control tumours (n=7), P=0.05.

Chapter 6

High Acute Myeloid Leukemia derived VEGFA levels are associated with a specific vascular morphology in the leukemic bone marrow

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Abstract

Background: Acute Myeloid Leukemia (AML) bone marrow biopsies at diagnosis display enhanced angiogenesis and increased VEGFA expression. In a xenograft mouse model it was described that availability of free VEGFA versus bound VEGFA is related to different vascular morphology. In this study we investigate the relationship between vascular morphology within AML bone marrow biopsies and AML derived VEGFA levels.

Methods: Vessel count and surface area (Chalkley count) were calculated in AML bone marrow biopsies at diagnosis (n=32), at remission (n=8) and Normal Bone Marrow (n=32) using immunohistochemical staining for FVIII, CD31, CTIV, SMA and VEGFA. VEGFA protein levels were measured.

Results: High vessel count was associated with an immature vessel status. Combining vessel count and Chalkley count different vessel morphology patterns were quantified within AML bone marrow biopsies. Three different subgroups could be distinguished. The subgroup (37.5% of the samples) exhibiting a high vessel count and vessels with predominantly large lumen (normal Chalkley count) was associated with high secreted VEGFA protein levels.

Conclusion: Different vasculature patterns are seen in AML bone marrow biopsies, defined by combining number and size of vessel. These quantified morphology patterns, combined with VEGFA levels, might be of value in the success of VEGF/VEGFR-signaling interference approaches.

Introduction

Acute Myeloid Leukemia (AML) is a disease with a poor outcome; the overall survival at 5 years is moderate to poor depending on age and cytogenetics [1-3]. At diagnosis an enhanced microvessel density (MVD) in bone marrow biopsies has been observed, which is restored to normal levels when a complete remission has been achieved [4,5]. In addition, in AML patients a high bone marrow blood flow and high tissue perfusion measured by MRI techniques is related to a significantly decreased disease-free survival and overall survival [6]. The enhanced bone marrow vascularization is correlated with an increased expression of Vascular Endothelial Growth Factor (VEGF) A [4]. Moreover, the level of VEGFA is an independent prognostic factor for treatment outcome in AML [4,7]. Clinical trials targeting the VEGF/VEGFR signaling pathway in patients with relapsed or refractory AML are reported and showed beneficial effects in a subset of patients. Treatment with Bevacizumab, a monoclonal antibody against VEGFA, following chemotherapy achieved a complete remission in 33% of patients with refractory AML [8]. A VEGF receptor tyrosine kinase inhibitor, SU5416, showed a significantly higher response rate and reduction of bone marrow microvessel density in patients with AML blasts expressing high levels of VEGF mRNA than in patients with low VEGF expression [9]. These reports show that there is at least heterogeneity in treatment response related to VEGFA levels.

Normal vasculature is highly organized and consists of vessels formed by Endothelial Cells (ECs) which are surrounded by basal membrane components and pericytes to provide structural and functional support [10]. This mature vasculature pattern is thought to be the result of a coordinated balance between pro-angiogenic and anti-angiogenic factors (reviewed in [11,12]). In contrast to the formation of normal blood vasculature, xenograft animal models showed that tumor vasculature is structurally and functionally abnormal; the hierarchical structure has been lost and dilated vessels and/or angiogenic vessel sprouts appeared [13-15]. The process of vessel formation comprises a disturbance in the balance of angiogenic factors, where VEGFA seems to play an important role [16]. These kind of differences in tumor vessel morphology seemed to be related to the availability of VEGFA; bound VEGFA resulted in angiogenic sprouting, whereas free or cleaved VEGFA resulted in dilated vessels in a xenograft mouse model [17].

In the present study we identified for the first time different structured networks of vessels in the bone marrow of newly diagnosed AML patients. Three different subgroups could be distinguished. In addition, one of the subgroups containing a high vessel count and a high vessel surface area was associated with high VEGFA protein level secreted by AML cells.

Materials and methods

Leukemic cells and bone marrow biopsy specimens

Bone marrow biopsies and leukemic cells of 32 newly diagnosed AML-patients were obtained at presentation after informed consent in accordance with the regulations and protocols sanctioned by the medical ethical committee. Clinical and cell biological data, including (cyto-)genetics as well as treatment outcome, were obtained from UMCG patient database. Table 1 summarizes patient characteristics of the AML patients. AML patients were treated following Dutch Hemato-Oncology Group (HOVON) protocols. In 8 AML patients a bone marrow biopsy was available at the time of complete remission. Normal control bone marrow biopsies were obtained from patients who underwent bone marrow biopsies as a staging procedure for a solid tumor or hematological insufficiency (e.g. anemia, leucopenia) and were diagnosed as normal (n=32). Mononuclear cells (MNCs) were separated using Lymphoprep (Nycomed, Oslo, Norway) density gradients and cryopreserved in liquid nitrogen until use. Cryopreserved AML cells were thawed rapidly at 37°C diluted in a 5x volume of normal calf serum (NCS) as previously described [18]. The remaining pellet was T cell-depleted by sheep red blood cells and separated over a Lymphoprep density gradient. The remaining blast cell population contained more than 95% AML cells and is hereafter referred to as AML cells.

Culture conditions for leukemic cells

AML cells were cultured at 1×10^6 cells/ml in X-vivo 10 medium supplemented with penicillin/streptomycin for 24h (FCS, Hyclone, Logan, UT, USA). Supernatants were harvested and centrifuged.

Detection of proteins using enzyme-linked immunosorbent assay (ELISA)

Secretion of human VEGFA by AML cells was detected in the supernatant using commercially available ELISAs (Quantikine immunoassays, R & D, Minneapolis, MN, USA) following the manufacture's instructions. VEGFA levels were detectable in 11 of the 30 samples.

RNA extraction and quantitative RT-PCR

Total RNA of the AML cells was extracted with the RNeasy mini kit according to the manufacturer's description (Qiagen, Breda, the Netherlands). An on column DNAse digestion was performed to purify the obtained RNA. cDNA was prepared by reverse transcription as described by the manufacturer (MBI Fermentas GmbH, St. Leon-Rot, Germany). The final reaction mix had a final volume of 20 µl and contained 1 µg total RNA, 1 µg random hexamers, 1mM of dNTPs, 20 U RNAse inhibitor and 200 U of RevertAid™ M-MuLV Reverse Transcriptase. Quantitative LightCycler PCR was performed using the LightCycler-FastStart DNA Master SYBR Green I system using 1 µl of a 10-fold diluted cDNA in each PCR reaction in a final volume of 10 µl (Roche Molecular Biochemicals, Mannheim, Germany). Specific primers for β_2-microglobulin were sense (CCA GCA GAG AAT GGA AAG TC) and antisense (GAT GCT TAC ATG TCT CG), PCR product 268 bp, 22 cycles, 55 °C; for VEGFA sense (GAG TGT GTG CCC ACT GAG GAG TCC AAC) and antisense (CTC CTG CCC GGC TCA CCG CCT CGG CTT), PCR product 177, 312, 384 bp, 32 cycles, 60 °C. PCR products were subjected

to melting curve analysis using the Light Cycler system to exclude the amplification of unspecific products. Finally, the PCR products were analyzed by conventional agarose gel electrophoresis. The expression of VEGF was standardized for expression of β_2-microglobulin (Arbitrary Units, AU). Serial cDNA dilutions of a mixture of all patient samples were used to generate standard curves. The expression of each gene in each sample was analyzed in duplicate. The regression coefficients of the standard curves were ≥0.99.

Table 1. Patient characteristics.

Characteristic	AML patients	Normal Bone Marrow	AML remission
No.	32	32	8
Age at diagnosis, y	54 (20-73)	58 (17-83)	48 (20-64)
Sex, male/female	17/15	17/15	4/4
White blood cell count (x 10^9/L)	28 (8-269)		
Peripheral blasts (%)	62 (3-97)		
Platelets (x 10^9/L)	48.7 (1.1-241.0)		
Cytogenetics, n (%)			
Favorable	2 (6)		
Intermediate	18 (56)		
Unfavorable	4 (13)		
Not available	8 (25)		
SCT			
No SCT	22		
Allogeneic SCT	7		
Autologous SCT	3		
Cycles to CR, n (%)			
1	13 (41)		
2	9 (28)		
3	2 (6)		
No CR	8 (25)		
Relapse, n (%)	18 (56)		
Dead/alive	24/8		
Staining			
Vessel count, n (%)	32 (100)	32 (100)	8 (100)
counts	16.2 (5.8-33.8)	7.4 (2.0-15.0)	5.7 (1.0-9.8)
Chalkley count, n (%)	32 (100)	32 (100)	8 (100)
counts	5.4 (3.2-11.0)	5.3 (3.3-10.3)	7.5 (6.0-10.0)
%CTIV-positive vessels, n (%)	29 (91)	28 (88)	7 (88)
counts	87.3 (2.0-100.0)	100.0 (0.0-100.0)	61.2 (0.0-100.0)
%SMA-positive vessels, n (%)	26 (81)	31 (97)	8 (100)
counts	35.0 (8.9-100.0)	73.1 (22.7-100.0)	54.9 (18.4-100.0)
VEGFA, n (%)	32 (100)	13 (41)	8 (100)

Characteristics—age, WBC, percentage peripheral blasts and platelets— and staining counts are given as median (range).

Immunohistochemical analysis

Bone marrow paraffin biopsy specimens were cut into 4-µm sections and stained for Factor VIII related antigen (FVIIIRA, also known as von Willebrand Factor), Collagen type IV (CTIV), CD31, SMA and VEGFA. Sections were deparaffined, blocked for endogenous peroxidase with 0.3% H_2O_2, blocked for antigen retrieval with 1% protease, immunostained for FVIIIRA (DAKO, AS, Glostrup, Denmark) and amplified with biotin–streptavidin HRPO (DAKO). The sections stained with CD31 (DAKO), SMA (DAKO) and CTIV (Southern Biotech, Birmingham, Alabama, USA) were treated the same way except for the antigen retrieval with 0.1 M Tris-HCl pH 9.0 at 80°C over night (SMA) or for 30 minutes in the microwave (CD31, CTIV). VEGFA (Santa Cruz, Heidelberg, Germany) stainings were blocked with 0.1 M Tris-HCl pH 9.0 for 15 minutes in the microwave and treated with an avidine/biotine block. The color reaction was assessed by adding substrate for peroxidase. After that the slides are colored with hematoxillin. Negative controls were produced by using non-specific IgG as the primary antibody (DAKO).

The vessel count measured by FVIIIRA was assessed using light microscopy in areas of the slide containing the highest numbers of blood vessels (hotspots). After the hotspots were identified, the total number of vessels per selected image was counted at ×400 magnification. At least five fields were counted for each section, and the true vessel number was expressed as the mean of five counts. Quantification of the relative area estimate of the vessels was assessed using the Chalkley point overlap morphometric technique as described before [19,20]. This method entails the use of an ocular grid with 25 random points. The ocular grid was turned to maximize the overlap between points on the grid and the vessels for five hotspots at 400x magnification. The number of overlapping points was counted for each of the hot spots, and the Chalkley count was expressed as mean value of the five counts. The Chalkley count was determined on FVIIIRA, CD31 and CTIV staining with a correlation coefficient of rho=0.714, p=0.001. Biopsies of 6 AML-patients and 1 normal bone marrow control could not be evaluated for SMA. Pericyte coverage (%SMA-positive vessels) and basal membrane coverage (%CTIV-positive vessels) were expressed as the percentage of FVIII positive vessels. The intensity of the VEGFA staining was analyzed semiquantitatively: 0=no staining, 1=slight staining, 2=moderate staining, 3=maximal staining. All stainings were evaluated by at least two separate investigators who had no knowledge of patient characteristics. Data was summarized in Table 1.

Statistical analysis

Statistical analysis was performed with PASW Statistics 18. The significance of changes in vessel counts, %SMA-positive vessels, VEGFA production and patient characteristics between three defined morphology groups or cytogenetic groups was assessed using non-parametric tests (Kruskal Wallis Test). The %SMA-positive vessels between two groups, and the correlation between vessel count and sex were determined with the non-parametric Mann Whitney-U test. Correlation between vessel count and patient characteristics was measured using the Spearman's rho. Correlation between vessel count and Chalkley count for the AML biopsies at diagnosis was measured using the Spearman's rho. A significant difference was defined as a p-value ≤ 0.05.

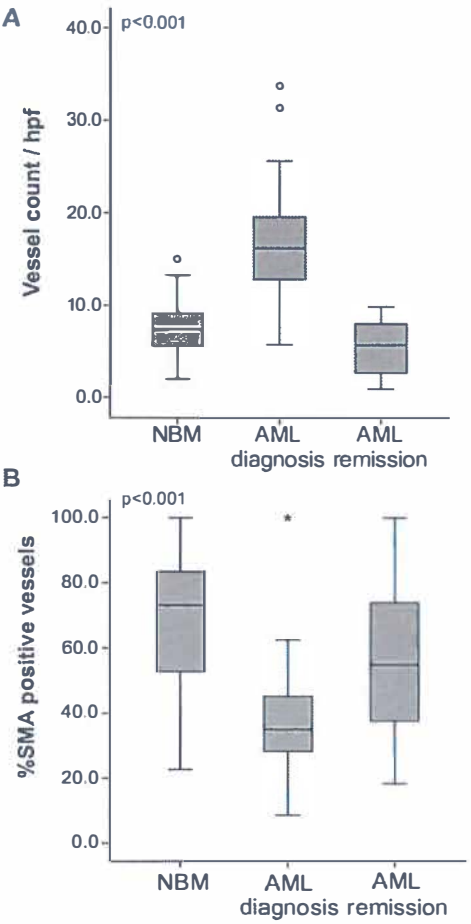
Results

Immature vessel status in AML bone marrow biopsies at diagnosis

Vessel count was significantly ($p<0.001$) increased in AML bone marrow at diagnosis (median: 16.2 microvessels/hpf, range 5.8-33.8, n=32) compared with normal control bone marrow (NBM, median: 7.4 microvessels/hpf, range 2.0-15.0, n=32), and returned to normal levels when complete remission was achieved (median: 5.7 microvessels/hpf, range 1.0-9.8, n=8) underscoring previous studies (Fig. 1A) [4,5]. Based on the fact that >95% of the Normal Bone Marrow biopsies and AML remission biopsies had a vessel count below 13 microvessels/hpf, it appeared that a higher vessel count was aberrant. Therefore, the value of 13 microvessels/hpf was taken as cut-off point for categorization into 'low vessel count' (13 or less) and 'high vessel count' (more than 13). Of the AML bone marrow biopsies 75% (24/32) were categorized as biopsies with a 'high vessel count'. No significant correlation was found between vessel count and patient characteristics such as WBC count ($p=0.65$, Spearman's rho: -0.08,), platelets ($p=0.85$, Spearman's rho: 0.04), blast percentage at diagnosis ($p=0.27$, Spearman's rho: -0.16), and age ($p=0.67$, Spearman's rho: 0.08), and no significant difference in vessel count was found for cytogenetics ($p=0.27$, Kruskal-Wallis test) and sex ($p=0.58$, Mann Whitney U test).

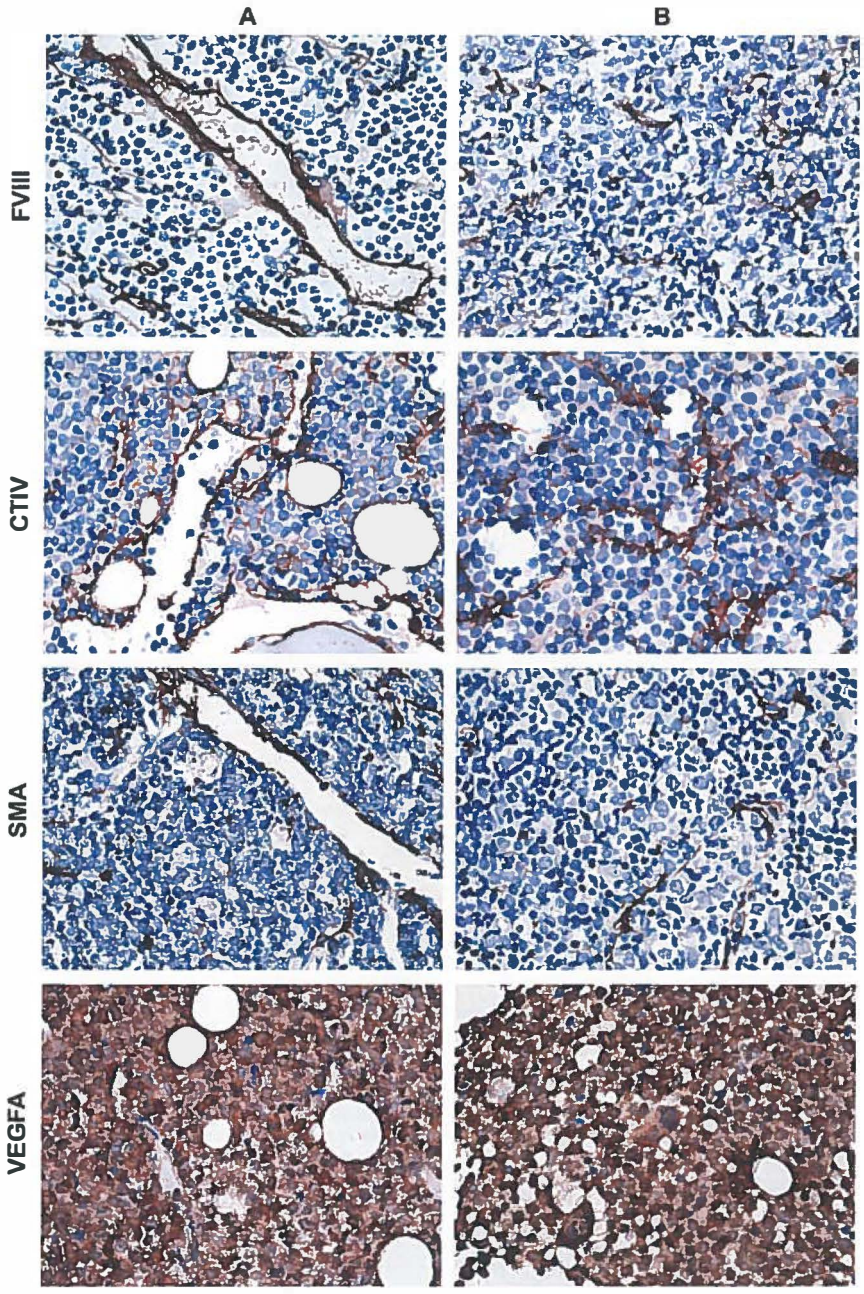
Basal membrane surrounding of the vessels was determined by the percentage collagen type IV (%CTIV) positive vessels. Median value in the AML bone marrow biopsies was 87.3% (range 2.1-100.0%, n=29) compared with 100.0% in the NBM (range 0.0-100.0%, n=28) and 61.2% in the AML remission bone marrow biopsies (range 0.0-100.0%, n=7). No significant difference was found between the groups, suggesting that the number of endothelial cell structures surrounded by basal membranes is comparable. Since vessel stabilization requires pericyte coverage of the vascular sprouts, we studied the number of vessels positive for Smooth Muscle Actin (SMA), a marker for pericytes. In AML bone marrow biopsies at diagnosis 35.0% of vessels were pericyte-coated (range 8.9-100.0%, n=26) versus 73.1% of vessels in the NBM (range 22.7-100.0%, n=31) and 54.9% in the AML remission group (range 18.4-100.0%, n=8) ($p<0.001$, Fig. 1B). Interestingly, the percentage vessels coated with pericytes was significantly ($p=0.04$) higher in the 'low vessel count' group (45%, range 23-100%, n=7) compared with the biopsies with 'high vessel count' (29%, range 9-57%, n=19), indicating that high vessel count in AML bone marrow is related to a more immature vessel status.

Figure 1. Bone marrow vessel count and pericyte coverage in AML.



(**A**) Boxplot of vessel count in AML bone marrow compared with normal bone marrow and AML remission. Vessel count in bone marrow biopsies of AML patients is significantly ($p<0.001$) higher than in NBM or AML remission group. Median values are represented by a bar in the boxplot. (**B**) Boxplot of percentage SMA-positive vessels in bone marrow biopsies of newly diagnosed AML patients, normal controls and AML patients in remission. Pericyte coverage is significantly ($p<0.001$) lower in bone marrow biopsies of AML patients at diagnosis. Median values are represented by a bar in the boxplot.

Figure 2. Morphology patterns in AML bone marrow biopsies.



Dilated vessels in panel A and sprouting vessels in panel B. Representative pictures of bone marrow immunohistochemistry for FVIII, CTIV and VEGFA (x400).

Different vasculature morphology patterns within AML bone marrow biopsies at diagnosis

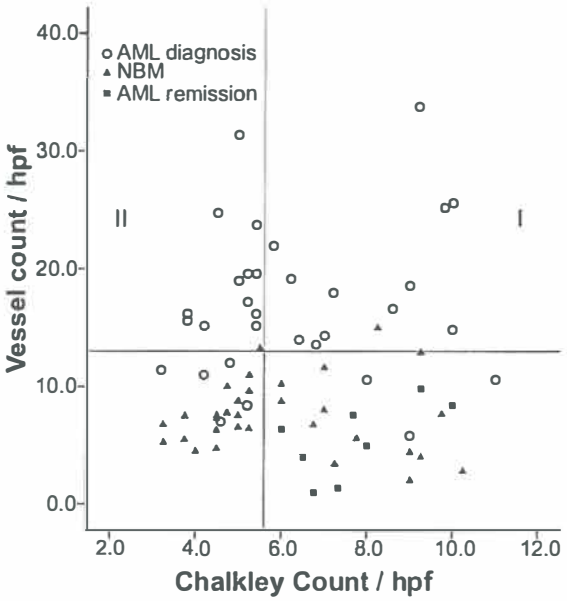
Assessing vessel morphology it appeared that different vasculature patterns within the AML bone marrow biopsies were seen (Fig. 2); biopsies displaying a high number of vessels with predominantly a large lumen and thin walls, but also biopsies exhibiting a high vessel count with mainly a network of small vessels with thin walls, narrow lumen and branching. To quantify this observation the vessel count was combined with a method to measure the vessel surface area, the Chalkley count; this is a quantification technique which determines a number of grid points that hit stained vessels or lumina. Figure 3 shows the results for vessel count and Chalkley count for the AML biopsies at diagnosis (median Chalkley count: 5.4, range 3.2-11.0), Normal Bone Marrow samples (median Chalkley count: 5.3, range 3.3-10.3) and AML at remission (median Chalkley count: 7.5, range 6.0-10.0). Within the group displaying a 'high vessel count' we further categorized the biopsies into two subgroups. The median value of 5.4 was taken as a cut-off for categorization into 'low' (5.4 or less) and 'high' (more than 5.4) Chalkley count. The first subgroup (12/32, 37.5%) had a high Chalkley count (group I in Fig. 3) and was defined as 'vessel hyperplasia', characterized by vessels with a predominantly large lumen and thin walls. The second subgroup (12/32, 37.5%) had a low Chalkley count (group II in Fig. 3) and was defined as 'angiogenic sprouting', displaying a network of small vessels with thin walls, narrow lumen and branching. Regarding patient characteristics the number of platelets at diagnosis was significantly (p=0.02) higher in the 'vessel hyperplasia' patients compared with the other two defined morphology patterns (data not shown). No differences were found between the morphological identified subgroups and other patient characteristics.

High free VEGFA levels excreted by AML cells related to vessel hyperplasia morphology

In a xenograft mouse model the availability of free versus bound VEGFA was related to the appearance of the tumor vasculature [17]. Since AML cells can secrete VEGFA we hypothesized that the observed differences in AML bone marrow vessel morphology could be related to excreted VEGFA protein levels. Immunohistochemical staining for total VEGFA protein expression showed a diffuse VEGFA distribution in all AML biopsies at diagnosis: the cytoplasm of leukemic blasts as well as the intercellular areas were mainly positive for VEGFA (Fig. 2). In contrast, Normal Bone Marrow biopsies showed a different distribution pattern, similar to biopsies of patients in complete remission: highly positive myeloid cells, a positive cytoplasm of megakaryocytes and erythroid cells that were negative for VEGFA (not shown). Immunohistochemistry (IHC) of the AML biopsies at diagnosis showed that the total amount of protein VEGFA was comparable in all samples. In accordance with the IHC the VEGFA mRNA level was not significantly (p=0.24) different within the three defined subgroups ('low vessel count' 3.1 AU, range 2.0-13.1; 'vessel hyperplasia' 6.1 AU, range 2.0-49.3; 'angiogenic sprouting' median 15.1 AU, range 2.6-64.6). In contrast, AML derived excreted VEGFA protein levels in AML at diagnosis were significantly (p=0.007) higher in the 'vessel hyperplasia' morphology subgroup compared to the 'angiogenic sprouting' subgroup and the 'low vessel count' biopsies (Fig. 4) (median 2.00 pg/ml, range 0-47.77, n=30). Overall, we conclude

that the total amount of VEGFA protein level and mRNA is similar in the AML biopsies at diagnosis whereas AML derived VEGFA levels are higher in the group displaying a 'vessel hyperplasia' pattern.

Figure 3. Scatterplot representing the biopsies of AML at diagnosis, at remission and Normal Bone Marrow.

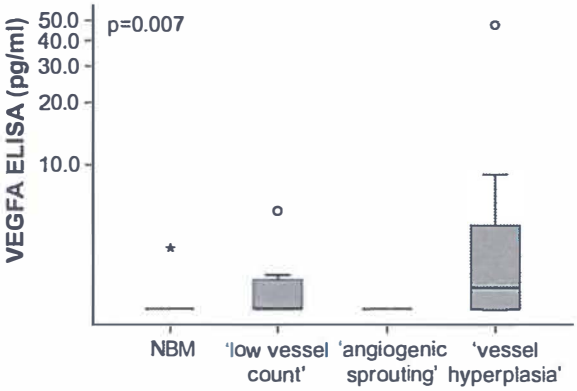


AML biopsies at diagnosis with a 'low vessel count' are displayed below the Y-axis reference line (13 microvessels/hpf), based on the fact that >95% of Normal Bone Marrow and AML remission biopsies had a vessel count below 13 microvessels/hpf. The X-axis reference line divides AML biopsies at diagnosis with a 'high vessel count' into two subgroups according to the median Chalkley count of 5.4 in AML at diagnosis and the identical cut-off point set by the median Chalkley count of the NBM and AML remission biopsies. Samples with a Chalkley count >5.4 were defined as 'vessel hyperplasia' (group I) and samples ≤5.4 as 'angiogenic sprouting' (group II). AML biopsies: Spearman's rho 0.15, p=0.42.

Discussion

Over the past years it has become clear that new vessel formation is part of the ongoing process in AML; MVD is increased in bone marrow biopsies of patients with newly diagnosed AML compared with normal bone marrow biopsies [4,5]. In our study we distinguished vessel heterogeneity in AML bone marrow biopsies at diagnosis. Using vessel count and Chalkley count we were able to divide the AML bone marrow biopsies at diagnosis into three subgroups: a subgroup with a 'low vessel count', a subgroup called 'angiogenic sprouting' (high vessel count, low Chalkley count) and a subgroup 'vessel hyperplasia' (high vessel count, high Chalkley count). To our knowledge, this is the first study that describes different quantified vasculature patterns in AML.

Figure4. Relation between AML derived VEGFA protein levels and vessel morphology in AML at diagnosis.



The AML-excreted VEGFA protein level was significantly ($p = 0.007$) higher in the subgroup with a 'vessel hyperplasia' morphology compared with the 'angiogenic sprouting' or the 'low angiogenic profile'. Box-and-whisker plot limits depict 75th and 25th percentiles and median value (box), and upper/lower quartile ± 1.5 × (interquartile range) (upper and lower whiskers, respectively).

In solid tumors, the morphology of vessels has been studied extensively. It shows that microvessels are not hierarchically structured and can consist of dilated vessels and/or angiogenic vessel sprouting [21]. These vasculature characteristics of solid tumors compromise the delivery and effectiveness of conventional cytotoxic therapies [22]. Treatment with VEGF/VEGFR-signaling interfering drugs induce a 'normalized' vessel pattern as described by vessel remodeling and increased pericyte coverage of tumor vessels [23,24]. As a result vessel stabilization and reduced permeability is achieved, facilitating a more efficient delivery of drugs to the targeted cancer cells. In our study we found that a high vessel count in AML bone marrow biopsies is related to a more immature vessel status, implicating that normalization of the vasculature might improve the outcome of chemotherapeutic drugs coadministered.

Drugs targeting VEGF/VEGFR signaling are promising in the treatment of cancer, with an immature vasculature within the tumor being more susceptible. This study shows that AML bone marrow biopsies have a high number of vessels, related to a low percentage of pericyte coverage. Recently, a clinical trial with Bevacizumab, a monoclonal antibody that binds VEGFA, following chemotherapy showed a favorable Complete Remission rate and duration in relapsed and refractory AML [8]. However, heterogeneity in the treatment response was seen during this trial. Since we describe differences in AML vasculature related to VEGFA levels the question raises: do different morphology patterns in AML bone marrow warrant distinct therapeutic approaches, thereby increasing the response? Ongoing work investigating the response of anti-VEGFA therapies related to AML bone marrow vasculature will have to answer this question.

In a xenograft mouse model the appearance of 'vessel hyperplasia' and 'angiogenic sprouting' was demonstrated and seemed to be related to the availability of VEGFA [17]. VEGFA can be released via breakdown of the Extracellular Matrix (ECM). This cleaved variant of VEGFA mainly produces dilated vessels. When VEGFA is not cleaved, and therefore not released from the ECM, it results in an angiogenic sprouting profile [17]. In corroboration with the abovementioned study we showed that biopsies with a high vessel count and a high Chalkley count were related to elevated AML blast derived VEGFA *in vitro* whereas 'angiogenic sprouting' and 'low vessel count' showed a relative low VEGFA level *in vitro*. These results support the view that the amount of free VEGFA could play a role in vascular patterning in AML. Interestingly, we also found that patients in the 'vessel hyperplasia' subgroup had a significantly higher platelet count at diagnosis compared with the other two defined subgroups. Since platelets can release VEGFA *in vivo*, an even higher VEGFA level can be expected enlarging vessel differences even more [25].

In conclusion, our study shows that heterogeneity in AML bone marrow vasculature can be quantified using Chalkley count in addition to vessel count and that biopsies displaying a high vessel count with low pericyte coverage could be diverted in two separate entities. Further studies will help to clarify its usefulness for adapting therapeutic approaches in trials with antiangiogenic or VEGFA-blocking drugs.

Acknowledgements

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Chapter 7

Patterns of bone marrow micro vessel morphology in Acute Myeloid Leukemia and high risk Myelodysplastic Syndrome predict treatment outcome following intensive chemotherapy and Bevacizumab

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Submitted.

Abstract

Background: Acute Myeloid Leukemia (AML) bone marrow biopsies at diagnosis display heterogeneity in angiogenesis, and can be divided into three distinct morphology groups as previously described, i.e. 'low vessel count', 'angiogenic sprouting', and 'vessel hyperplasia'. In this study we investigate the relationship between vascular morphology within bone marrow biopsies and clinical outcome in HOVON81, a multicenter phase II trial that evaluated the effect of Bevacizumab added to standard chemotherapy in elderly AML patients.

Methods: AML bone marrow biopsies at diagnosis (n=93) were immunohistochemical stained for FVIII and SMA. Vessel count and surface area (Chalkley count) were calculated and biopsies were divided into three groups. Groups were compared, among others, by Kaplan-Meier curves. Univariate and multivariate Cox-regression analysis was performed.

Results: This larger cohort confirmed the three previously reported AML vessel morphology patterns. A decreased EFS was seen for patients with 'angiogenic sprouting' and 'low vessel count' compared with 'vessel hyperplasia' (P=.017). There was a trend toward difference in OS (P=.055), with a worse outcome for 'angiogenic sprouting'. In multivariate analysis, the variable 'morphology groups' was established as independent prognostic factor for EFS and OS.

Conclusion: These results show that vessel morphology in AML bone marrow biopsies has a prognostic value. Vasculature morphology patterns might be a useful tool to predict which patients will benefit from additional anti-angiogenic treatment strategies to the standard treatment.

Introduction

Angiogenesis is involved in the progression of multiple malignancies, including Acute Myeloid Leukemia (AML). Bone marrow biopsies of AML patients at diagnosis display an enhanced microvessel density (MVD), restoring to normal vessel counts when a complete remission has been achieved [1,2]. Moreover, increased bone marrow angiogenesis in AML measured by MRI could predict adverse clinical outcome [3,4]. The enhanced MVD is correlated with increased expression of Vascular Endothelial Growth Factor A (VEGFA), which is found to be a prognostic factor for therapeutic outcome in AML [1,5-7]. Within AML bone marrow biopsies at diagnosis different vasculature patterns are appreciated, and three subgroups could be distinguished, i.e. a subgroup with 'low vessel count', a subgroup called 'angiogenic sprouting' (biopsies exhibiting a high vessel count with mainly a network of small vessels with thin walls, narrow lumen and branching) and a subgroup 'vessel hyperplasia' (biopsies displaying a high number of vessels with predominantly a large lumen and thin walls) [8].

Drugs targeting VEGF/VEGFR-signaling has proven clinical benefit in patients with solid malignancies [9-11]. In AML, a phase II clinical trial of Bevacizumab, a monoclonal antibody against VEGFA, administered after chemotherapy to 48 adults with refractory or relapsed AML demonstrated an overall response rate of 48% with a complete remission rate of 33% [12]. In addition, a marked decrease in microvessel density was observed after Bevacizumab treatment. A subsequent second study with nine relapsed or refractory AML patients investigated single agent Bevacizumab in AML, and did not display any significant anti-leukemic activity despite a reduction in VEGFA expression in the bone marrow [13]. All together, although data are rather scarce, VEGF/VEGFR-signaling interference studies report heterogeneity in AML treatment response.

Recently, HOVON81, a multicenter phase II trial, evaluated the effect of Bevacizumab added to standard induction chemotherapy in more than 200 patients with AML and high risk MDS above 60 years. In the total group of patients, no advantage for the addition of Bevacizumab could be detected (unpublished data). In the present study we examined whether bone marrow vessel patterns could be related to outcome and treatment response to Bevacizumab in the HOVON81 trial. We showed that the previously reported distinct AML vessel morphology patterns could be observed in this larger cohort of AML/MDS patients: 'low vessel count', 'angiogenic sprouting' and 'vessel hyperplasia'. Interestingly, we found that patients displaying 'vessel hyperplasia' showed a favorable Event Free Survival (EFS) compared with the other two subgroups, whereas the subgroup 'angiogenic sprouting' vessel pattern had a worse Overall Survival (OS) compared with 'vessel hyperplasia' and 'low vessel count'.

Materials and methods

Patients and leukemic bone marrow biopsy specimens

HOVON81 trial is a multicenter randomized phase II trial for elderly patients (age >60 years) with a confirmed diagnosis of AML (except those with FAB M3) or high risk MDS (defined as refractory anemia with excess of blasts (RAEB) or refractory anemia with excess of blasts in transformation (RAEB-T) with an International Prognostic Score System (IPSS) score ≥ 1.5 and a WHO performance score (PS) of 2 or less. Secondary AML progressing from antecedent myelodysplasia was also eligible. Patients received Daunorubicin 45 mg/m2 on day 1-3 and Cytarabin 200 mg/m2 on day 1-7. Bevacizumab was administered in a dosage of 5 mg/kg or 10 mg/kg i.v. in 60 minutes at day 1 and 15 of cycle I and II. Bone marrow biopsies were performed before cycle I and between day 28-35 after cycle I. Eligible patients who have given written informed consent in accordance with the regulations and protocols sanctioned by the medical ethical committee were registered and randomized before start of treatment. Bone marrow biopsies of 104 AML-patients prior to treatment were obtained. From 53 AML-patients a bone marrow biopsy after Cycle I was available. Clinical and cell biological data, including (cyto)genetics as well as treatment outcome, were obtained from the HOVON data centre. Patients were classified into prognostic categories on the basis of the karyotype of the leukemic cells. Favourable risk was defined by the presence of abnormalities in core-binding factors; unfavourable risk, by the presence of complex cytogenetic abnormalities (at least three unrelated cytogenetic abnormalities), monosomies or partial deletions of chromosome 5 or 7 (del(5q), del(7q), -5, -7), abnormalities of the long arm of chromosome 3 (q21;q26), t(6;9)(p23;q34), t(9;22)(q34;q11.2), or abnormalities involving the long arm of chromosome 11 (11q23) unless the criteria for a monosomal karyotype were fulfilled; very unfavourable risk, by the presence of a monosomal karyotype. Any other cytogenetic abnormalities, as well as AML without cytogenetic abnormalities or with loss of an X or Y chromosome as the only abnormality, were considered to indicate an intermediate risk [14]. Table 1 summarizes patient characteristics of the AML patients.

Immunohistochemical analysis

Bone marrow paraffin biopsy specimens were cut into 4-µm sections and stained for Factor VIII related antigen (FVIIIRA, also known as von Willebrand Factor) and SMA (Smooth Muscle Actin). Sections were deparaffined, blocked for endogenous peroxidase with 0.3% H_2O_2, blocked for antigen retrieval with 1% protease, immunostained for FVIIIRA (DAKO, AS, Glostrup, Denmark) and amplified with biotin–streptavidin HRPO (DAKO). The sections stained with SMA (DAKO) were treated the same way except for the antigen retrieval with 0.1 M Tris-HCl pH 9.0 at 80°C over night (SMA). The color reaction was assessed by adding substrate for peroxidase. After that the slides are colored with hematoxillin. Negative controls were produced by using non-specific IgG as the primary antibody (DAKO). As previously described, the vessel count measured by FVIIIRA was assessed using light microscopy in areas of the slide containing the highest numbers of blood vessels (hotspots) at ×400 magnification. All stained cells or cell clusters, with or without lumen, separate from other stained structures were counted as microvessels.

Table 1. Patient characteristics

Characteristic	All patients	Low vessel count	Angiogenic sprouting	Vessel hyperplasia	p-value
No.	93	19	33	41	
Age at diagnosis, y	66 (60-79)	67 (61-72)	67 (61-77)	65 (60-79)	.568
Sex, male/female	53/40	10/9	21/12	22/19	.629
White blood cell count (x 10^9/L)	3.1 (0.5-212.9)	3.1 (0.5-117)	2.6 (0.9-212.9)	5.3 (0.8-194.0)	.749
Platelets (x 10^9/L)	62 (6-403)	73 (6-320)	68 (6-403)	53 (17-279)	.489
WHO performance score					.306
0	51	11	18	22	
1	37	7	12	18	
2	4	0	3	1	
Not available	1	1	0	0	
Cytogenetics, n (%)					.145
Favorable	3 (3.2)	2 (10.5)	1 (3.0)	0 (0.0)	
t(8;21)	2	1	1	0	
inv(16)	1	1	0	0	
Intermediate	62 (66.7)	14 (73.7)	22 (66.7)	26 (63.4)	
normal karyotype	44	8	17	19	
+8	6	2	1	3	
-X/Y	3	1	1	1	
other	9	3	3	3	
Unfavorable	24 (25.8)	2 (10.5)	10 (30.3)	12 (29.3)	
11q23	1	0	0	1	
complex	3	0	2	1	
-5/7q	10	2	3	5	
t(6;9)	2	0	1	1	
other	8	0	4	4	
Not available	4 (4.3)	1 (5.3)	0	3 (7.3)	
CR, y/n	64/29	11/8	20/13	33/8	.096
1 cycle to CR	48 (51.6)	9 (47.4)	16 (48.5)	23 (56.1)	
2 cycles to CR	16 (17.2)	2 (10.5)	4 (12.1)	10 (24.4)	
No CR	29 (45.3)	8 (42.1)	13 (39.4)	8 (19.5)	
Dead/alive	68/25	14/5	26/7	28/13	.598
Bevacizumab treated, n (%)	42 (45.2)	9 (47.4)	16 (48.5)	17 (41.5)	.814
5 mg/kg	7 (16.7)	1 (11.1)	2 (12.5)	4 (23.5)	
10 mg/kg	35 (83.3)	8 (88.9)	14 (87.5)	13 (76.5)	

Characteristics - age, WBC, percentage peripheral blasts and platelets—are given as median (range). P-value of 'low vessel count' versus 'angiogenic sprouting' versus 'vessel hyperplasia'.

The true vessel number was expressed as the mean of four counts. In these four hotspots quantification of the relative area estimate of the vessels was assessed using the Chalkley point overlap morphometric technique as described before [15]. In short, an ocular grid with 25 random points was turned to maximize the overlap between points on the grid. The number of overlapping points was counted for each of the hotspots, and the Chalkley count was expressed as mean value of the four counts. As previously reported, AML biopsies were divided into three groups based on the fact that >95% of Normal Bone Marrow biopsies and AML remission biopsies had a vessel count below 13 microvessels/hpf and the median Chalkley count of the AML biopsies was 5.4. The three subgroups: biopsies with 'low vessel count' (<13 microvessels/hpf), biopsies with 'angiogenic sprouting' (≥13 micovessels/hpf, Chalkley count ≤5.4), or biopsies as were defined as 'vessel hyperplasia' (≥13 micovessels/hpf, Chalkley count >5.4). Pericyte coverage (%SMA-positive vessels) was expressed as the percentage of FVIII positive vessels. Biopsies of 11 AML-patients could not be evaluated. SMA staining was evaluated in 67 biopsies (low vessel count n=18, angiogenic sprouting n=17, vessel hyperplasia n=32). All stainings were evaluated by at least two separate investigators who had no knowledge of patient characteristics and outcome.

Statistical analysis

Statistical analysis was performed with PASW Statistics 18. Quantitative parameters differences between the defined morphology groups were evaluated using a Student t test for normally distributed variables or a Kruskal-Wallis Test for skewed distributed variables, and using the Chi-square test for the contingency tables. Correlations were calculated with the Spearman Rank correlation coefficient (rho). Actuarial probabilities of OS (with death resulting from any cause) and EFS (with failure in case of no complete remission or relapse or death) were estimated according to the Kaplan-Meier method. The Kaplan-Meier curves were compared by using a long-rank statistic test. Univariate cox analysis was used to determine the association between EFS/OS and vessel morphology groups (low vessel count, angiogenic sprouting or vessel hyperplasia), treatment with Bevacizumab (yes or no), AML or MDS, sex (male or female), initial white blood cell (WBC) count (i.e. $<20x10^9/l$ or $>20x10^9/l$), age at diagnosis (i.e. 60-65 years, 66-70 years or >70 years), cytogenetics (due to small numbers divided into two groups, i.e. favorable and intermediate versus unfavorable), and WHO performance score (due to small numbers divided into two groups, i.e. 0 versus 1 and 2). Multivariate cox regression analysis was applied to test the association between the morphology groups and OS/EFS with adjustment for the significant risk factors; the variable cytogenetics was included in the multivariate cox regression analysis (P=.051). All tests were two-tailed and a significant difference was defined as a p-value ≤ 0.05.

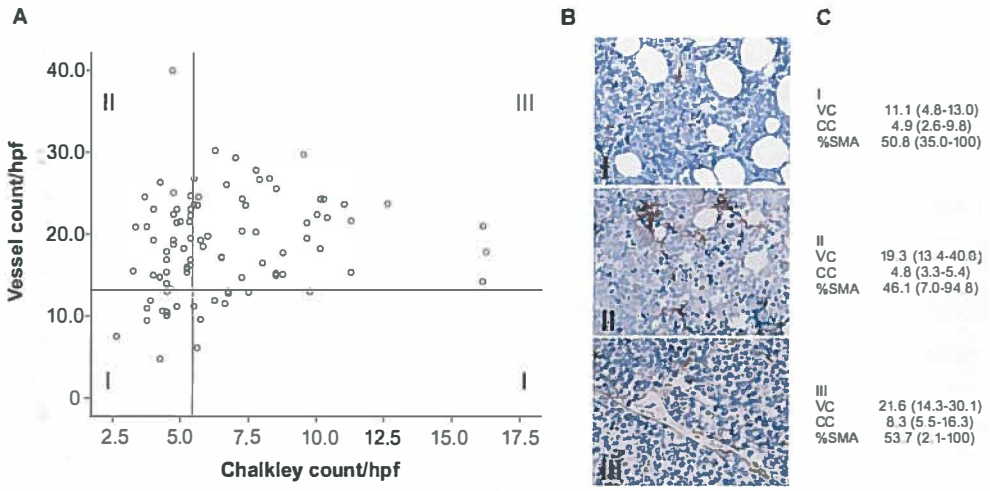
Results

Three distinct vasculature patterns within AML/MDS bone marrow biopsies at diagnosis

In a previous study we have found three different vasculature patterns in the bone marrow of newly diagnosed AML patients [8]. To validate these morphology patterns in a larger cohort, AML bone marrow biopsies of 93 newly diagnosed AML/MDS patients were screened for Vessel count (VC) and Chalkley

count (CC) in FVIII stained slides. Median value of VC was 18.8 microvessels /hpf (range 4.8-40.0, n=93) and the median value of CC was 5.6 (range 2.6-16.3, n=93); these counts are in concordance with our earlier results [8]. Figure 1A shows the results for VC and CC with cut-off values as previously described, resulting in the following groups: biopsies with a 'low vessel count' (<13 microvessels/hpf, n=19, Group I), biopsies displaying 'angiogenic sprouting' (a high vessel count with mainly a network of small vessels with thin walls, narrow lumen and branching, n=33, Group II), and biopsies exhibiting 'vessel hyperplasia' (a high number of vessels with predominantly a large lumen and thin walls, n=41, Group III) (Figure 1A-B). The median percentage of vessels covered by pericytes was 50.5% (range 2.1-100.0, n=67), and was not significantly (P=.18) different between the three subgroups (%SMA-positive vessels, Figure 1C). No significant differences were found between the results presented here and the data reported in our previous work[8].

Figure 1. AML biopsies prior to treatment divided into three morphology groups.



(**A**) Scatterplot representing the AML biopsies at diagnosis. Biopsies with a 'low vessel count' are displayed below the Y-axis reference line (13 microvessels/hpf, group I). The X-axis reference line divides AML biopsies at diagnosis with a 'high vessel count' into two subgroups according to the median Chalkley count of 5.4 in AML at diagnosis. Cut-off points are based on previously described results. Samples with a Chalkley count ≤5.4 were defined as 'angiogenic sprouting' (group II) and samples with a Chalkley count >5.4 as 'vessel hyperplasia' (group III). (**B**) A representative picture of the three groups is shown. (**C**) Staining counts for Vessel count (VC), Chalkley count (CC) and %SMA-positive vessels (%SMA) are given as median (range).

Vessel morphology patterns in AML/MDS bone marrow biopsies at diagnosis related to treatment outcome

To study the clinical relevance of the distinct vessel morphology patterns within adult AML bone marrow biopsies, the association between clinical parameters and the distinct morphology patterns was evaluated. No significant correlation was found between morphology pattern and patient characteristics including WBC count, platelets, percentage peripheral blasts and age. Also cytogenetics and sex were not significantly different among patients with distinct vasculature morphology patterns (Table 1).

Interestingly, a significant decreased EFS was seen for patients with 'angiogenic sprouting' and 'low vessel count' compared with 'vessel hyperplasia' (P=.017, Figure 2A). For OS, a trend was seen with a worse outcome for the angiogenic sprouting subgroup (P=.055, Figure 2B). Together, patients displaying an 'angiogenic sprouting' profile seem therefore to constitute an unfavorable subset of patients.

Cox univariate analysis showed that besides vessel morphology groups, also WBC at diagnosis showed significant association with EFS, whereas age at diagnosis, treatment with Bevacizumab, AML or MDS, sex, and WHO performance score and cytogenetics did not affected EFS. OS was significantly affected by the variables cytogenetics and morphology groups. Treatment with Bevacizumab, age at diagnosis, AML or MDS, sex, WHO performance score, and WBC at diagnosis did not significantly associate with OS (data not shown). Next, we included the significant variables (i.e. WBC at diagnosis, cytogenetics and vessel morphology groups) in a multivariate cox regression analysis. The variables vessel morphology group and WBC count were established as independent prognostic factors for EFS (vessel hyperplasia: HR: 2.112, 95% CI: 1.216-3.676, P=.008; WBC < $20x10^9$/l: HR: 1.745, 95% CI: 1.028-2.964, P=.039, Table 2). For OS, the variables vessel morphology groups, cytogenetics, and WBC count had an independent prognostic value (vessel hyperplasia HR: 2.104, 95% CI: 1.189-3.723, P=.011; Favorable/intermediate: HR: 1.953, 95% CI: 1.095-3.485, P=.023; WBC <$20x10^9$/l: HR: 1.793, 95% CI: 1.031-3.119, P=.039, Table 2).

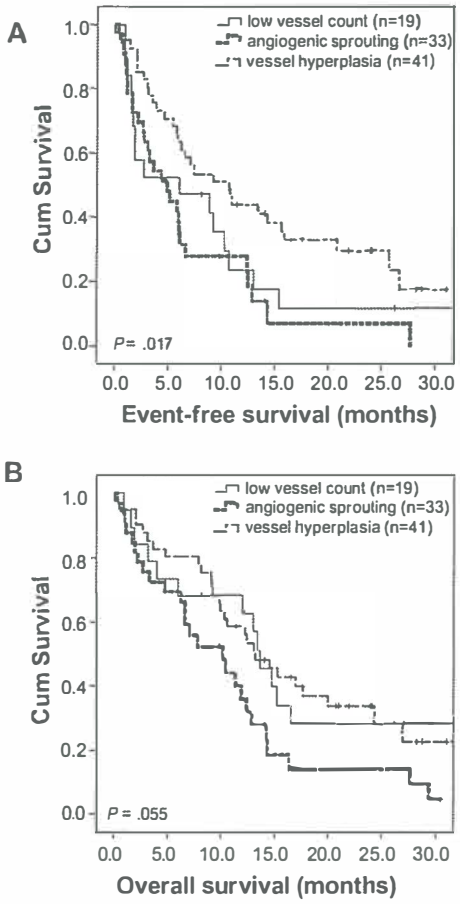
Table 2. Multivariate analysis of morphology groups, WBC count and cytogenetics as a prognostic factor for EFS and OS.

	Event Free Survival		Overall Survival	
Variable	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Low vessel count†	1.264 (.664-2.408)	.476	1.942 (.958-3.936)	.065
Vessel hyperplasia†	2.114 (1.216-3.676)	.008	2.104 (1.189-3.723)	.011
Favorable/intermediate*	1.581 (.916-2.728)	.100	1.953 (1.095-3.485)	.023
WBC‡	1.745 (1.028-2.964)	.039	1.793 (1.031-3.119)	.039

HR: hazard ratio; CI: confidence interval; WBC: white blood cell count. Cytogenetics: cytogenetic risk group as defined in 'Material and Methods'.

† Morphology group versus 'angiogenic sprouting'. * Favorable/intermediate versus unfavorable. ‡ WBC < $20x10^9$/l versus WBC > $20x10^9$/l.

Figure 2. Vessel morphology patterns in relation to event-free survival and overall survival.



Kaplan Meier plots show (**A**) EFS and (**B**) OS of AML patient subgroups divided by defined vasculature patterns within the bone marrow biopsies, i.e.'low vessel count' (n=19), 'angiogenic sprouting' (n=33), and 'vessel hyperplasia' (n=41).

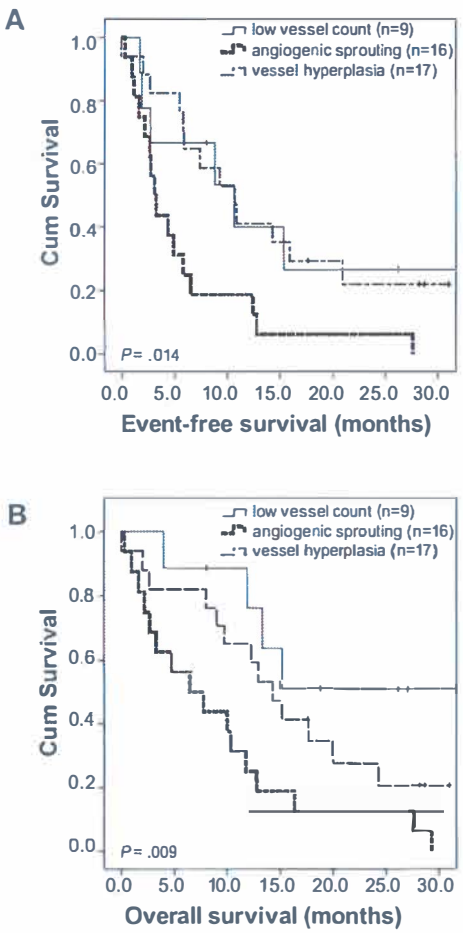
Treatment with Bevacizumab might affect outcome in patients displaying a low vessel count

Within the group of patients treated with Bevacizumab the clinical parameters such as age at diagnosis, WBC count, cytogenetics and sex of the three vessel morphology subgroups were uniformly distributed (n=42, data not shown). The number of Bevacizumab-treated patients that achieved CR did not significantly differ between the three vessel morphology groups (P=.235). As found in the whole group, within the groups of patients treated with Bevacizumab EFS and OS were significantly (P=.014 versus P=.009) decreased in patients displaying an 'angiogenic sprouting' pattern compared with the other two subgroups (Figure 3A-B). Noteworthy, when considering only patients treated with 10mg/kg Bevacizumab (35/42, 83%), the vessel morphology group 'angiogenic sprouting' still showed a

significantly lower EFS and OS (P=.008 and P<.001 respectively, data not shown).

Next, we analyzed the effect of Bevacizumab treatment on CR, EFS and OS within the three defined vessel morphology groups. Within the vessel morphology subgroups, the number of patients achieving CR was not significantly affected by treatment with Bevacizumab (data not shown). Although our results are limited by the small number of patients displaying 'low vessel count', treatment with Bevacizumab significantly increased EFS and tended to be associated with a beneficial OS (P=.023 and P=.099 respectively, n=9 versus n=10 patients,). For the 'angiogenic sprouting' or 'vessel hyperplasia' profiles no effect of Bevacizumab could be detected (data not shown).

Figure 3. Vessel morphology patterns in relation to event-free survival and overall survival within the patient group treated with Bevacizumab.



Kaplan Meier plots show (**A**) EFS and (**B**) OS of the AML patient treated with Bevacizumab. The subgroups are divided by defined vasculature patterns within the bone marrow biopsies: 'low vessel count' (n=9), 'angiogenic sprouting' (n=16), and 'vessel hyperplasia' (n=17).

Changes in vasculature patterns within AML bone marrow biopsies after Cycle I

Since detection of a change in vessel counts after Bevacizumab treatment may be a method to measure Bevacizumab activity (i.e. VEGFA blockade activity) *in vivo*, we determined the vasculature in AML bone marrow biopsies after the first treatment cycle. Serial samples prior to treatment and after Cycle I were evaluable from a total of 44 patients. Of these 44 patients, 25 already received CR after Cycle I whereas 19 patients achieved CR after Cycle II or did not achieve CR. Moreover, of these 44 evaluable patients, 22 were treated with Bevacizumab added to standard chemotherapy. No significant correlation was found between changes in VC or CC and patient characteristics, addition of Bevacizumab, and/or all treatment parameters (data not shown).

To note, shifts between the three defined morphology groups after Cycle I occur. After cycle I, 17 of 44 (39%) of patients are assigned to different morphology groups as before treatment (data not shown), suggesting that morphology analysis should occur before start of treatment. Shifts in morphology groups are not associated with Bevacizumab addition or survival parameters (data not shown).

Discussion

Assessing the number of vessels in AML bone marrow biopsies is a well-known and commonly used method to describe the degree of *in vivo* vascularization [1,16]. However, in view of the observed heterogeneity in AML vasculature we previously described a method to quantify morphological appearance of the AML bone marrow vasculature by combining the number of vessels (i.e. vessel count) with a method to measure the vessel surface area (i.e. Chalkley count) [8]. In this study we demonstrate that the previously reported three AML vessel morphology patterns (i.e. 'low vessel count', 'angiogenic sprouting' and 'vessel hyperplasia') could be appreciated in an independent larger cohort by combining vessel count and Chalkley count.

Cytogenetics, age, and performance status have long determined prognosis and therapy in AML, where cytogenetics remain the most important disease-related prognostic factor. In our study, cytogenetics was an independent prognostic factor for OS, whereas it did not have a significant value for EFS. This might be due to the small number of patients, or relapse treatment. Age and performance score did not significantly affected OS and EFS. Interestingly, the variable 'vessel morphology groups' was an independent prognostic factor for EFS and OS.

The clinical outcome of the three distinct morphology groups showed a worse outcome for patients with an 'angiogenic sprouting' profile. We hypothesize that the survival disadvantage of the 'angiogenic sprouting' group, characterized by the most aberrant vessel pattern with a high number of small vessels, might result in an inadequate delivery and effectiveness of therapeutics due to impaired blood supply and elevated pressure interfering with the delivery of the drugs [17]. A 'normalized' vessel pattern as described by vessel remodeling would facilitate a more efficient delivery of drugs.

Enhanced angiogenesis in AML bone marrow biopsies at diagnosis is correlated with raised VEGFA levels, which is an independent prognostic factor for clinical outcome [5-7]. Thus, targeting VEGFA may be a promising approach for inducing a clinical response in AML patients, in particular for patients displaying high vessel counts. In this study, addition of Bevacizumab yielded favorable EFS for patients with a 'low vessel count', although in small numbers, whereas no beneficial effect was seen for AML patients displaying a high vessel count, i.e. 'angiogenic sprouting' and/or 'vessel hyperplasia'. This observation raised the question whether Bevacizumab was sufficient to neutralize circulating VEGFA levels of 'angiogenic sprouting' and 'vessel hyperplasia' groups. Two clinical studies investigated the effect of Bevacizumab 10 mg/kg, and showed decreased serum levels of VEGFA during treatment [12,13]. Another functional assay to measure VEGFA blockade activity *in vivo* might be detection of a change in vessel count. Our results showed no decrease in vasculature counts after one cycle of Bevacizumab. In a smaller group, Karp et al. showed a marked decrease in vessel count in 73% of the patients (8/11, of whom 5 achieved CR) after administration of a single dose Bevacizumab 10 mg/kg, supporting the notion that the ability to interfere with VEGF/VEGFR signaling may be relevant to the clinical activity of Bevacizumab [12]. Whether Bevacizumab 10 mg/kg was sufficient to neutralize VEGFA in patients displaying a high vessel count in our study has not been studied.

Response to anti-angiogenic drugs might also be related to maturity of the tumor vasculature; Helfrich and colleagues evaluated human melanoma metastases taken at clinical relapse in patients undergoing adjuvant treatment using Bevacizumab. Tumor vessels resistant to anti-VEGF therapy were characterized by enhanced vessel diameter and normalization of the vascular bed by coverage of pericytes [18]. We did not find a relationship between response to Bevacizumab and maturity of the vasculature (i.e. pericytes-coverage).

Heterogeneity in treatment response to therapies that target VEGF are described, and it seems difficult to find validated biomarkers for selecting patients who will benefit from addition of those drugs, i.e. Bevacizumab [19]. We attempted to evaluate whether vessel morphology in AML bone marrow might be associated with treatment response. Although our results are limited by the small number of patients displaying 'low vessel count', statistical analysis did reveal significant patterns for EFS, with a trend for OS. It is possible that results for OS would have emerged with larger numbers.

In conclusion, our study shows that the observed heterogeneity in AML bone marrow vasculature is related to outcome. Assessing vascular morphology patterns in AML bone marrow biopsies might be a promising biomarker to define which patients will benefit from treatment with additive anti-angiogenic treatment strategies and should be further evaluated in controlled clinical trials.

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Chapter 8

Summary, general discussion and future perspectives

Summary

Acute Myeloid Leukemia (AML) is a hematological disorder, characterized by a disturbed differentiation of the myeloid lineage. Currently the main treatment for AML is chemotherapy, followed by stem cell transplantation for a subgroup of patients. Although the outcome of AML has improved during the last decades, over 40% of the AML patients experience a relapse, while the relapse rate depends on the age and cytogenetics. Therefore, new therapeutic strategies are still warranted.
In (pediatric) AML, cellular and circulating levels of Vascular Endothelial Growth Factor A (VEGFA) are elevated and are adversely associated with prognosis [1-3]. Co-expression of VEGFA and its tyrosine kinase receptors VEGFR1 and VEGFR2 has been reported on AML cells, where VEGF/VEGFR-signaling has proliferative effects on the AML cells [4,5]. Moreover, increased VEGFA levels are associated with a higher microvessel density in the bone marrow of newly diagnosed AML patients [6,7]. Together, the data suggest that targeting VEGF/VEGFR signaling might be an interesting therapeutic implication. The aim of this thesis was to explore the various mechanisms of VEGFA on AML tumor progression via autocrine and paracrine mechanisms, including angiogenesis. We investigated the potential implications of VEGF/VEGFR interference in targeting AML.

Angiogenesis, the formation of new blood vessels, is a very complex and tightly regulated process. In solid tumors, this multistep process is disturbed and contributes to tumor growth and metastatic dissemination. Current studies show that angiogenesis is also involved in the pathogenesis of hematological malignancies. Having been first described in the 1980s, VEGFA is defined as one of the key players in angiogenesis. The importance of VEGFA production and/or new vessel formation in hematological malignancies resulted in efforts to target angiogenesis. Therapeutic drugs designed to inhibit these processes include the inhibition of angiogenic factors (e.g. VEGF), the blockade of their receptors, anti-endothelial cell strategies and vascular targeting. Preclinical and clinical trials are performed in patients with hematological malignancies and indicate that these drugs are safe and offer potential clinical utilities. In **chapter 2** we reviewed the current data on angiogenesis and VEGFA in hematological malignancies, and describe the results of anti-angiogenic strategies in these diseases.

PTK787/ZK 222584 (PTK/ZK) is a tyrosine kinase inhibitor targeting VEGFR2 and VEGFR1, and to a lesser extent VEGFR3, PDGFRß, c-KIT, and c-FMS. In **chapter 3** we used total cell kill assays to show that treatment with PTK/ZK induced cell death in three AML cell lines, whereas a VEGFR2-negative leukemic cell line was unaffected by PTK/ZK. Besides, PTK/ZK reduced cell survival in pediatric AML samples. Since chemotherapeutic drugs are considered the standard therapy for AML, we investigated the additional effect of PTK/ZK upon cell death achieved by the chemotherapeutic drug Amsacrine. In both cell lines and patient samples, the dosage of Amsacrine could be lowered and replaced by the potentially less toxic PTK/ZK to achieve equal amounts of AML cell death.

AML is thought to be initiated and maintained by a subset of self-renewing Leukemic Stem Cells (LSC), also called Leukemia Initiating Cells (LICs), residing in the CD34+ cell fraction enriched for LICs. We questioned whether VEGF and VEGFR signaling might play a role in the outgrowth of these LICs. To study the concept of the LIC, a leukemic long-term culture initiating cells (LTC-IC) assay has been described in which long-term leukemic expansion of leukemic progenitor/stem cells can be established using MS5 bone marrow stromal cells that mimic the so called stem cell niche [8]. Using the LTC-IC assay we demonstrated in **chapter 4** that pediatric AML stem/progenitor cells can be cultured in this assay, with a long-term expansion of leukemic cells up to 10 weeks of culture in 9 of 13 samples. Addition of PTK/ZK to pediatric LICs resulted in a reduction in leukemic expansion in all samples ranging from 4-80% growth at week 5 compared with the untreated samples. In 75% of the samples the effect was more pronounced at the end of the culture (week 10). FACS analysis of the CD34+ sorted pediatric AML cells was performed and showed that VEGFR2 protein expression could be detected in 11 of the 13 AML samples, ranging from 3% to 94% VEGFR2 positive cells. VEGFR3, VEGFR1, c-Kit, PDGFRβ and c-FMS showed various expression levels. Data on mRNA level showed that expression of most of these RTKs was present in the AML cells although in different expression profiles. No correlation was found between the responsiveness to PTK/ZK and the protein or mRNA expression level of one of the receptors in particular. Using proteome profiler arrays we demonstrated that PTK/ZK reduced activation of PI3K/Akt kinase signaling. To investigate whether the response of LICs to PTK/ZK could be attributed to VEGFA/VEGFR signaling, we transduced MS5 stromal cells with a retroviral vector containing VEGFA. We demonstrated that the outgrowth of LICs was not affected when exposed to stroma-derived VEGFA or in the presence of Bevacizumab (monoclonal antibody against VEGFA), suggesting that the effect of PTK/ZK cannot be assigned to specific inhibition of VEGFA/VEGFR signaling. In conclusion, our data elucidated the anti-leukemic properties of PTK/ZK, and suggest that targeting multiple RTKs by PTK/ZK might be a potential effective approach in eradicating the primary pediatric AML cell.

Besides autocrine effects, VEGFA secreted by the AML cells can stimulate surrounding endothelial and stromal cells in a paracrine fashion. As previously described, addition of VEGFA to endothelial cells induced cytokine expression from these cells in a dose-dependent way *in vitro* [9]. Consequently, an increase in stromal and endothelial cell derived growth factors may contribute to the growth of the leukemic cells. To study the role of VEGFA promoting tumor growth, we used HL-60 cells transduced with VEGFA165 or control vector using retroviral constructs (**chapter 5**). In *in vitro* cultures no benefit of VEGFA165 overexpression was could be detected. The interaction of VEGFA with its environment was investigated by s.c. injecting VEGFA165 cells or control cells in NOD/SCID mice. In contrast to the *in vitro* results, VEGFA overexpression *in vivo* resulted in a clear increase in tumor weight and immunohistochemical analysis showed an enhanced tumor cell proliferation and increased angiogenesis. To obtain a more detailed understanding of the phenotype within the tumor cells, we performed gene expression profiling on the tumors. Within the list of genes differentially expressed between both tumor groups, a number of human genes involved in the TGF-ß signaling pathway were listed, i.e. the TGFBR2 was lower expressed

in the VEGFA165 tumors whereas the inhibitory SMAD7 was higher expressed within the VEGFA165 tumors. Since VEGFA165 overexpression did not confer proliferative advantages *in vitro*, these data suggest a downregulation of the TGF-ß signaling pathway in the tumor cells via a paracrine route. To further investigate the VEGFA-driven paracrine effects on the host-derived cells, we performed mouse-specific cytokine arrays and found an increased expression of the cytokines IFNG and IL7, both known to induce SMAD7 expression. Together, these data suggest a role for stromal interaction in VEGFA induced tumor growth via inhibition of the TGF-ß signaling pathway.

In AML bone marrow biopsies at diagnosis an enhanced angiogenesis is displayed, related to high VEGFA levels. The morphology of the increased bone marrow vasculature seemed widely varying. In **chapter 6** we studied angiogenesis in bone marrow biopsies of AML patients at diagnosis, in remission and Normal Bone Marrow controls by immunohistochemical staining for endothelial cells (FVIII), the basal membrane (CTIV) and pericytes (SMA), and confirmed an increased number of vessels in AML at diagnosis. We demonstrated that a high vessel count was associated with an immature vessel status (i.e. low percentage SMA-positive vessels). Within the AML bone marrow biopsies at diagnosis three morphological phenotypes could be appreciated, and quantification of these observations was assessed by combining the vessel count with the Chalkley count, i.e. the vessel surface area: a subgroup with a 'low vessel count' (low vessel count, variable Chalkley count), a subgroup called 'angiogenic sprouting' displaying a network of small vessels with thin walls, narrow lumen and branching (high vessel count, low Chalkley count) and a subgroup 'vessel hyperplasia' characterized by vessels with a predominantly large lumen and thin walls (high vessel count, high Chalkley count). Since it was described that availability of free VEGFA versus bound VEGFA is related to different vascular morphology in a xenograft mouse model, we hypothesized that the observed differences in vascular morphology was related to VEGFA levels. AML derived VEGFA protein levels were higher in the 'vessel hyperplasia' subgroup.

Heterogeneity in AML vasculature raised the question if different morphology patterns are related to patient characteristics and/or clinical parameters such as outcome. In **chapter 7** we made use of AML bone marrow biopsies of patients included in a randomized clinical trial of Bevacizumab in addition to standard chemotherapy (HOVON81). Bone marrow biopsies at diagnosis (n=93) were immunohistochemical stained for FVIII, and vessel count as well as Chalkley count was calculated. Again the three distinct morphology patterns could be detected, i.e. 'low vessel count', 'angiogenic sprouting' and 'vessel hyperplasia'. No significant correlation was found between morphology pattern and patient characteristics. Analysis of the clinical parameters showed that patients displaying 'angiogenic sprouting' profile and 'low vessel count' showed significant decreased EFS compared with 'vessel hyperplasia'. There was a trend toward a difference in OS (P=.055), with a worse outcome for the subgroup 'angiogenic sprouting'. Multivariate analysis defined the variables vessel morphology group and WBC count as independent prognostic factors for EFS (vessel hyperplasia: HR: 2.112, 95% CI: 1.216-3.676, P=.008; WBC < $20x10^9$/l: HR: 1.745, 95% CI: 1.028-2.964, P=.039). The variables vessel morphology group, cytogenetics

and WBC count were established as independent prognostic factors for OS (vessel hyperplasia HR: 2.104, 95% CI: 1.189-3.723, P=.011; Favorable/intermediate: HR: 1.953, 95% CI: 1.095-3.485, P=.023; WBC <20x10^9/l: HR: 1.793, 95% CI: 1.031-3.119, P=.039). In addition, analysis of Bevacizumab treatment within the three defined vessel morphology groups revealed that addition of Bevacizumab to standard chemotherapy significantly affected EFS for patients with 'low vessel count', although groups were small. These results suggest that vasculature patterns might be a useful tool to predict AML outcome in general, with a possible role for Bevacizumab treatment in patients with a 'low vessel count'.

General discussion and future perspectives

AML and stroma

The role of the microenvironment in cancer development is being increasingly appreciated. Interaction of tumor cells with their immediate tumor microenvironment support the development of both tumor cells and non-malignant cells. Leukemic cells reside within the bone marrow, where the bone marrow stromal cell population consists of fibroblasts, adipocytes, endothelial cells and osteoblasts. *In vitro* and *in vivo* studies show that several components of the bone marrow microenvironment, including endothelial cells and osteoblasts, contribute to enhanced leukemic cell survival [10,11]. In addition, stromal cells can protect AML cells from chemotherapy-induced apoptosis [12,13]. These findings suggest that disrupting leukemic-stromal cell interactions might be essential to eradicate AML. A more profound understanding of the leukemic-stromal cell interaction will elucidate the mechanisms involved in the progression of leukemia. In order to study the role of tumor-derived VEGFA on the outgrowth of tumor cells in interaction with its environment, we subcutaneously injected AML cells transduced with VEGFA or a control vector (chapter 5). Crosstalk between AML cells and mouse-derived stromal cells resulted in an increased tumor weight of tumors derived from VEGFA-cells, accompanied by (i) increased tumor cell proliferation, (ii) enhanced angiogenesis, (iii) increased production of host-derived cytokines and (iv) downregulation of genes involved in the TGF-ß signaling pathway within the tumor cells. Future studies are required to further unravel the interaction of stromal-leukemic cells in the bone marrow; more specifically, investigating the interference with the TGF-ß signaling pathway as a potential target in the treatment of AML.

Targeting the Leukemia Initiating Cell by stroma and stroma-derived factors

Therapy surviving LICs are thought to be responsible for relapses of AML patients. Targeting these cells might, therefore, improve survival rates. At the time of (pediatric) AML diagnosis, the levels of VEGFA protein in the cell, plasma and supernatant are an independent prognostic factor for disease-free and overall survival, CR and overall survival, and relapse-free survival respectively [13]. We studied the effect of the tyrosine kinase receptor inhibitor PTK787/ZK 222584 (PTK/ZK; targeting VEGFR2, VEGFR1, VEGFR3, PDGFRß, c-KIT, and c-FMS) on the outgrowth of pediatric LICs in a LTC-IC assay (chapter 4). Adding PTK/ZK to the cultures inhibited leukemic expansion of the pediatric CD34+ sorted AML cells. However, no

significant effect was seen when LICs were cultured in the presence of Bevacizumab, a monoclonal VEGFA-antibody. In AML, deregulation of signal transduction pathways may promote leukemogenesis by conferring cell proliferation and survival advantages. Activation of single signal transduction pathways, such as the Ras/Raf/MAPK/ERK, PI3K/Akt/mTOR and Jak/STAT pathways, has an adverse effect on AML outcome [14-16]. Simultaneous activation of multiple signal transduction pathways is common in AML, and survival progressively decreases as the number of activated pathways increases [17]. These downstream intracellular signaling pathways are mainly activated by phosphorylation of RTKs. Whether the effect of PTK/ZK can be assigned to the inhibition of one specific receptor or ligand, or is the result of multiple receptor tyrosine kinase inhibitions, remains unanswered. It is likely that leukemia treatment targeting multiple signal transduction pathways may be more efficacious than therapy aimed at inhibiting a single pathway.

Hematopoietic Stem Cells (HSCs) reside within specialized 'stem cell niches' in the bone marrow, which can be defined as two different niches: the 'osteoblastic niche' and the 'vascular niche' [18]. The osteoblastic niche is located near the bone surfaces in the bone marrow and provides a microenvironment for long-term quiescence of the HSC [19]. In turn, the vascular niche consists of sinusoidal endothelium lining blood vessels, and promotes proliferation and differentiation of cycling HSCs [20,21]. It is thought that the LICs also reside in these bone marrow niches. In the assay described in this thesis, this microenvironment is mimicked by MS5 stromal cells, and provides a model in which LICs can be cultured and studied. An interaction between the stromal cells and the LICs, cultured on these cells, can occur. We cannot exclude a direct effect of PTK/ZK on stromal cells. However, we did not see phenotypical changes in MS5 stromal cells when cultured with PTK/ZK.

It is noteworthy to state that the long-term leukemic stem/progenitor assays described in chapter 4 were all performed in normoxic conditions. However, recent reports in mice indicate that the endosteum at the interface of bone and bone marrow is thought to be hypoxic [22]. Experimental studies involving co-cultures of primary AML cells and stromal cells (MS5) in hypoxic conditions should help to clarify the influence of low oxygen levels on the outgrowth of primary pediatric AML cells. Moreover, exposure to low oxygen concentrations induces the expression of hypoxia-inducible factor-1 alpha (HIF1α). A well-described function of HIF1α is upregulation of VEGFA [23,24]. How the hypoxic conditions affect the treatment response to the tyrosine kinase inhibitor and changes in VEGFA exposure has not been studied yet.

Vascular heterogeneity in AML

Angiogenesis is a process described to contribute to growth and progression of multiple malignancies, including hematological malignancies [7,25]. In AML, secretion of different angiogenic growth factors and mediators (e.g. VEGFA) by leukemic blasts and stromal cells both contribute to new vessel formation [4,26]. Vascular heterogeneity occurs in AML bone marrow biopsies at diagnosis. In this thesis we set out to investigate whether different vessel morphology patterns could be defined, and whether these morphology patterns might have different outcome rates. We distinguished three different subgroups

of structured vessel networks in the bone marrow of newly diagnosed AML patients, i.e. a subgroup with 'low vessel count', a subgroup called 'angiogenic sprouting' (biopsies exhibiting a high vessel count with mainly a network of small vessels with thin walls, narrow lumen and branching) and a subgroup 'vessel hyperplasia' (biopsies displaying a high number of vessels with predominantly a large lumen and thin walls). We found that the subgroup containing high vessel count and high vessel surface area (i.e. vessel hyperplasia) was associated with high VEGFA protein level secreted by AML cells (chapter 6). Interestingly, chapter 7 demonstrates that patients displaying an 'angiogenic sprouting' or 'low vessel count' profile had a worse EFS compared with the subgroup 'vessel hyperplasia', whereas OS tended to be significant (P=.055), with a worse outcome for the subgroup 'angiogenic sprouting'.

Animal studies demonstrated that vascular morphology patterns might be related to the availability of free or bound VEGFA. Soluble/non-bound VEGF is made by either (1) alternative exon splicing or (2) proteolytic processing. The human VEGFA gene, located on chromosome 6p12, includes eight exons separated by seven introns [27,28]. As a result of alternative exon splicing of mRNA, at least nine different isoforms of varying amino acid lengths can be produced. The splice variants differ in biological properties: whereas the smaller isoforms lack a heparin-binding domain (located in exons 6 and 7) and are soluble secreted proteins, the larger forms are almost completely bound to heparin-containing proteoglycans in the cell surface or extracellular matrix (ECM). VEGFA 165 is the predominant human splice variant and has intermediate properties as it is secreted, but a significant fraction also remains bound to the cell membrane [29,30]. Grunstein et al studied the role of distinct VEGFA isoforms on tumorigenic neovascularization, and showed that tumors of the splice variant VEGF188 in mice (termed VEGF189 in human, i.e. matrix-bound) displayed an extensive hypervascular response with small convoluted vessels in comparison with the other isoforms [31]. Subsequently, Ruhrberg et al showed that mouse embryos expressing solely the heparin-binding VEGF188 isoform ($VEGF^{188/188}$) exhibited a phenotype with ectopic branching structures in the form of long and thin microvessels, resembling our 'angiogenic sprouting' pattern. In contrast, microvessels from mouse embryos expressing predominantly or solely the non-heparin-binding VEGFA isoforms ($VEGF^{wt/120}$ or $VEGF^{120/120}$ respectively), showed that nascent endothelial cells were preferentially integrated within existing vessels to increase lumen caliber, resembling our 'vessel hyperplasia' pattern [32]. Together, these studies imply that specific expression of each isoform result in alterations in vascular morphology. Interestingly, we found that in AML bone marrow biopsies the subgroup containing high vessel count and high vessel surface area (i.e. the subgroup 'vessel hyperplasia') was associated with higher free VEGFA protein levels. Therefore, we can hypothesize that the observed vessel morphology might be the result of differentially expressed splice variants. However, in AML, co-expression of four VEGFA isoforms (i.e. VEGF121, VEGF165, VEGF183, and VEGF189) was shown [33], suggesting that the expression of different VEGFA splice variants does not seem to regulate vascular morphology patterns in AML bone marrow biopsies.

Soluble VEGF can also be made by proteolytic processing, resulting in the release of the receptor-binding domain from the ECM-binding motif of the protein. Lee et al showed that a subset of Matrix

metalloproteinases (MMPs) can cleave matrix-bound isoforms of VEGF, releasing soluble fragments [34]. In addition, they described that introduction of an MMP-cleaved VEGFA isoform in a xenograft mouse model (i.e. free VEGFA) resulted in capillary dilation of existent vessels. In contrast, the expression of MMP-resistant VEGF was matrix-bound and supported extensive growth of thin vessels with multiple and frequent branching points, resembling our 'angiogenic sprouting' pattern. Whether the expression of MMPs is related to the AML vessel morphology patterns found in our study has not been studied.

Response to drugs targeting VEGF/VEGFR-interference

Angiogenesis is a critical stromal process in tumor growth and development and, therefore, targeting angiogenesis might be an important target for anticancer therapy. At this moment, the U.S. Food and Drug Administration has approved four drugs that interfere with the VEGF/VEGFR pathways for the treatment of various solid tumors including colorectal cancer, renal cell cancer and non small cell lung cancer. Bevacizumab (Avastin; Genentech) was the first anti-angiogenic therapy approved, a monoclonal antibody against all isoforms of VEGFA. This drug works by binding to VEGFA, thereby preventing the binding of VEGFA to its receptor. Karp et al demonstrated that chemotherapy followed by Bevacizumab yields a favorable CR rate (in 33% of the patients) and overall response (48%) in adults with AML that are resistant to traditional treatment approaches [35].

The other group of approved anti-angiogenic drugs includes three receptor tyrosine kinase (RTK) inhibitors, Sunitinib (Sutent, SU11248; Bayer), Sorafenib (Nexavar; Bayer), and Pazopanib (Votrient; GlaxoSmithKline). These drugs bind to the tyrosine kinase inhibitor, preventing the phosphorylation of the receptor, finally resulting in inhibition of downstream signaling pathways. Clinical trials in AML patients show that results of these drugs are comparable with outcome data from studies using Bevacizumab. Monotherapy with Sunitinib (inhibitor for fms-like tyrosine kinase 3 (FLT3), c-KIT, VEGFRs, and PDGFR) induced partial remissions of short duration in a phase 1 study for patients with refractory AML [36].

Sorafenib (inhibitor for Raf kinases, FLT3, PDGFR, VEGFRs) in combination with clofarabine and cytarabine has been tested in a phase I study for 12 relapsed/refractory pediatric AML patients, of which six patients (three FLT3-ITD and three FLT3 wild-type AML) achieved complete remission [37]. In a phase I/II study of combination therapy with sorafenib, idarubicin, and cytarabine in 51 patients with acute myeloid leukemia (< 65 years), a CR rate of 75% was demonstrated, where FLT3-mutated patients were more likely to achieve CR than FLT3-wt patients [38]. In addition, five phase I studies tested monotherapy Sorafenib before or after Stem Cell Transplantation in adult AML patients with relapsed and refractory AML, and confirmed the beneficial effects of Sorafenib on FLT3-mutated AMLs [39-43]. To our knowledge, Pazopanib has not been clinically tested in AML. The drug used in our studies, PTK/ZK, has also been tested in a phase I study for treatment of primary refractory or relapsed AML. Treatment with induction chemotherapy and PTK/ZK resulted in a complete remission (CR) in 5 of 17 patients, suggesting its potential clinical benefit [44].

While targeting the host 'tumor-supporting' angiogenic processes has many benefits, it might also have limitations. Recent data highlight the importance of understanding the effect that anti-angiogenic agents cause within the tumors. When Sunitinib was used as short-term and sustained monotherapy in orthotopically implanted tumors in mice, antitumor effects were found. However, when mice were treated before intravenous implantation of tumor cells or following removal of primary grown tumors, acceleration of metastasis was observed, followed by a decreased survival [45]. In addition, another preclinical study reported that treatment of tumor-bearing mice with single agent anti-VEGFR2 antibody or Sunitinib elicited increased local progression of tumors and enhanced growth of metastasis after prolonged treatment [46]. Together, these studies show that anti-angiogenic drugs have potent effects on tumor growth, but also show that the same anti-angiogenic drug can have distinct effects on tumor growth when administered in different time schedules or different tumors. An explanation for this mechanisms of resistance VEGF/VEGFR-inhibition include an upregulation of compensatory growth factors, e.g. by stromal cells [47]; regrowth of vessels after cessation of treatment in which pericytes might have an important role [48]; and mobilization of bone-marrow derived cells that might facilitate and enhance growth of tumors and metastasis [49-52]. To note, in the aforementioned reports monotherapy was used. Future studies are warranted to gain more insight into the optimal treatment regimes of VEGF/VEGFR-interfering drugs, e.g. duration of the treatment, continuous treatment or intermittent treatment, neoadjuvant or adjuvant strategies, and the combination of anti-angiogenic drugs, with or without other anti-cancer drugs including chemotherapeutic drugs.

Another approach could be normalization of tumor vasculature. Treatment with VEGF/VEGFR-signaling interfering drugs induces a 'normalized' vessel pattern as described by vessel remodeling and increased pericyte coverage of tumor vessels [53]. As a result vessel stabilization and reduced permeability is achieved, which might facilitate a more efficient delivery of drugs to the targeted cancer cells.

Potential biomarkers for treatment with VEGF/VEGFR-interfering drugs

VEGFA/VEGFR2-signaling interference studies report heterogeneity in treatment response. Therefore, biomarkers are needed to predict which patients will benefit from VEGFA/VEGFR2-interfering drugs. At this moment current studies focus on the detection of new biomarkers to optimize further study designs.

Tumor-specific markers and vascular morphology - In search of new predictive biomarkers, Koeppen and colleagues tested multiple tumor-specific markers to predict the treatment effect of Bevacizumab in metastatic colorectal cancer. Tumor VEGF expression, thrombospondin-2, or mutations of KRAS, BRAF or p53 did not predict benefit from treatment with Bevacizumab [54,55].

Since VEGF/VEGFR2 signaling is the main signaling pathway implicated in new vessel formation, tumor angiogenesis in response to VEGF/VEGFR interfering drugs has also been subject of multiple studies. Whereas most studies did not define microvessel density (MVD) as a significant biomarker for treatment response, Yang et al showed a significant decrease in MVD after Bevacizumab treatment, related to treatment outcome in patients with breast cancer [55].

Helfrich and colleagues evaluated the vascular network in spontaneously developing melanomas of MT/*ret* transgenic mice after using PTK787/ZK222584, and analyzed human melanoma metastases taken at clinical relapse in patients undergoing adjuvant treatment using Bevacizumab. In both models they found that tumor vessels that are resistant to anti-VEGF therapy were characterized by enhanced vessel diameter and normalization of the vascular bed by coverage of pericytes [56]. In our study we did not find a relationship between response to Bevacizumab and pericyte-coverage of the vasculature or other vessel markers (chapter 7). Interestingly, we demonstrate that addition of Bevacizumab to standard chemotherapy significantly affected survival rates for one of the three defined vessel morphology patterns, i.e. 'low vessel count' morphology. Although our results are limited by the small number of patients displaying 'low vessel count', AML patients displaying 'low vessel count' might be candidate for Bevacizumab treatment in the future.

Circulating markers – As the main cytokine targeted in anti-angiogenic drugs, multiple studies investigated the prognostic value of circulating VEGFA levels for treatment response to Bevacizumab. Even though some trials have shown promising results, a meta-analysis of four phase III studies showed that measurement of baseline circulating VEGF levels may be useful as a prognostic biomarker (information about the patients overall cancer outcome, regardless of treatment), but not as a predictive biomarker (the probable effect of a particular therapeutic intervention) for Bevacizumab-based treatment benefit in metastatic colorectal, lung, and renal cell cancers [57]. However, free VEGF levels were significantly decreased after the start of Bevacizumab therapy, suggesting that Bevacizumab reduced the plasma level of biologically active VEGF [58]. In contrast to what one would expect from treatment with anti-VEGF antibodies or VEGFR inhibitors, other studies show an increase in VEGFA levels after treatment, restoring to basic levels when drug administration has been stopped [59,60]. The explanation for these inconsistent reports might be the difficulties and differences in the detection of VEGFA levels: VEGFA can be present in different isoforms, VEGFA can be released by platelets during preparation and measurement, VEGFA might be free or bound in the circulation, and it is unclear how to interpret the results of the measurements.
Alterations in the concentration of other angiogenic proteins in relation to VEGF/VEGFR interfering drugs have been described, and so far only circulating levels of intercellular adhesion molecule 1 (ICAM-1) was shown to have a predictive value for better one-year survival and overall survival after treatment with Bevacizumab in patients with non-small cell lung cancer [61]. Together, further extensive analysis on identifying a predictive biomarker is needed to expand on this work.

Hypertension - Given the association between hypertension and improved survival rates during Bevacizumab treatment in clinical trials from solid tumors, hypertension was suggested to be a useful marker of VEGFA activity [62-64]. Binding of VEGF to VEGFR2 upregulates production of nitric oxide by endothelial cells and/or production of the paracrine vasodilator prostacyclin and leads to local vasodilatation and reduced blood pressure [65,66]. Bevacizumab inhibits VEGFA signaling, and

hypothetically, hypertension therefore predicts the anti-angiogenic activity of this drug [67]. These studies, however, raise the question whether an adverse effect should be used as a biomarker. Nevertheless, the finding suggests that patients experiencing hypertension may have greater potential to gain significant therapeutic effect. An aggressive treatment of the Bevacizumab-induced hypertension may therefore provide more options compared with abandoning the therapy due to toxicity.

Imaging – Although tumor response to conventional chemotherapy has been traditionally measured through reduction of tumor size, different anti-angiogenic therapies may necessitate new modalities in measuring treatment response, e.g. to determine the effect of these drugs on tumor vasculature. Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) is a noninvasive technique for assessment of microvascular structures with tracking of the pharmacokinetics of injected low-molecular-weight contrast agents as they pass through the tumor vasculature [68]. In AML, increased bone marrow angiogenesis as measured by DCE-MRI predicted adverse clinical outcome [69,70]. Remarkably, vertebral bone marrow perfusion parameters (e.g. high peak slope, high amplitude, low elimination rate) as measured by DCE-MRI in AML patients in complete remission, were associated with shorter relapse free survival and overall survival [71]. Up to now the association between DCE-MRI and the treatment response to anti-angiogenic therapies in AML has not been described. In patients with breast cancer treated with chemotherapy plus Bevacizumab a greater reduction in tumor perfusion by DCE-MRI was seen compared with patients treated without Bevacizumab, although there was no correlation with outcome parameters [72,73].

Another method for early assessment of treatment response in patients with AML is the use of positron emission tomography (PET) scans. By means of PET it is possible to visualize the hypoxic regions and metabolic activity. Eight AML patients receiving induction chemotherapy underwent whole-body ^{18}F-fluorothymidine (FLT)-PET/CT scans, and showed that both during and after therapy, AML patients who entered complete remission displayed bone marrow with low FLT uptake in contrast to higher uptake in those patients with resistant disease [74]. In patients with biliary-tract cancers, changes in ^{18}F-fluorodeoxyglucose (FDG)-PET after two cycles of Bevacizumab showed a significant decrease in maximum standardized uptake value, which was associated with disease control and increased PFS and OS [75]. Together, these findings show that DCE-MRI and/or PET may help to monitor treatment response for tailored anti-angiogenic therapy in AML. In light of our results, it would be very interesting to combine the observed different vasculature patterns in AML bone marrow biopsies with imaging techniques. Another option would be to label the drug and measure its distribution as well as its binding to the target, i.e. VEGFA. Nagengast et al. were the first to demonstrate that radiolabeled Bevacizumab can be used as a tracer for noninvasive in vivo imaging of VEGF in the tumor microenvironment; tumor uptake determined by quantification of small-animal PET images was higher for radiolabeled-Bevacizumab compared with control [76]. Recently, in a clinical study VEGF-SPECT with radiolabeled-Bevacizumab could visualize all known melanoma lesions in stage III/IV melanoma patients, suggesting VEGF presence in the lesions. A single Bevacizumab dose lowered uptake of radiolabeled-Bevacizumab [77]. Although these techniques are still in the early stages of testing, the results seem promising.

Polymorphisms – Another possible biomarker that might predict treatment efficacy is evaluation of Single Nucleotide Polymorphisms (SNPs). Polymorphisms in VEGFA and VEGFR2 have been proposed to predict benefit from Bevacizumab treatment. The association between Bevacizumab efficacy and VEGFA genotypes was studied in a clinical trial for advanced breast cancer patients, and showed that -2578AA and 1154AA genotypes predicted a favorable survival [78]. Two smaller studies did not find any association with response to Bevacizumab for the VEGFA SNPs [79,80]. These findings pose the question: what is the potential biological mechanism underlying these findings? Possible explanations have been described, including lower VEGFA tumor expression in -2578AA and 1154AA genotypes, decreased VEGFA serum levels in carriers of the 2578A allele, or a decreased MVD in patients with -2578CC genotype [78,81,82]. These studies suggest a role for VEGFA SNPs in relation to treatment response to Bevacizumab. However, the number of patients studied is small and the results are variable.

Summarizing, the need for biomarkers is critical with increasing numbers of anti-angiogenic agents being approved. At this moment no validated biomarkers for selecting patients who will respond to anti-angiogenic therapy are available. However, preliminary biomarker data are emerging and a number of biomarkers need a more extensive analysis to prove the clinical usefulness [83].

Conclusions

Despite the progress that has been made during the past decades in the therapy of AML, a considerable part of children and adults still die from their disease. Therefore, significant efforts toward improving the clinical outcome of AML patients are still needed.

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Chapter 9

Nederlandse samenvatting

Nederlandse samenvatting

Hematopoiese

In het menselijk lichaam worden ongeveer 1 biljoen nieuw bloedcellen per dag aangemaakt, een proces dat hematopoiese wordt genoemd. Dit is nodig omdat de bloedcellen een beperkte levensduur hebben, en daardoor steeds vervangen moeten worden door nieuwe bloedcellen. In het beenmerg (de rode stof in de holle ruimte van botten) zit een beperkt aantal cellen dat daarvoor verantwoordelijk is, de stamcellen. Deze stamcellen zijn in staat om zichzelf te delen, en twee cellen te vormen; een van beide cellen zal een rustende stamcel blijven, de tweede cel zal een ander celtype worden door deling (proliferatie) en uitrijping (differentiatie). Deze tweede jonge voorlopercel kan zich uiteindelijk ontwikkelen tot rode bloedcel (erythrocyt; belangrijk voor het vervoeren van zuurstof en CO2 in het bloed), witte bloedcel (leukocyt; belangrijk voor de afweer) of bloedplaatje (trombocyt; spelen een rol in het stollen van bloed) (zie ook figuur 1 van hoofdstuk 1). De gevormde volwassen bloedcellen zullen vanuit het beenmerg de bloedbaan betreden, en daar hun functie uitoefenen.

Acute Myeloïde Leukemie

Wanneer de normale hematopoiese verstoord raakt, kan er leukemie (bloedkanker) ontstaan. Leukemie is een verzameling van ziektes waarbij de jonge voorlopercellen zich niet volledig ontwikkelen tot een rijpe bloedcel, maar blijven steken in de ontwikkeling: ze delen wel, maar rijpen niet voldoende uit. Acute Myeloïde Leukemie (AML) is een vorm van leukemie, en kan ontstaan uit de 'myeloïde voorlopers', de voorlopercellen voor rode bloedcellen, bloedplaatjes of een bepaald soort witte bloedcellen. Bij leukemie vermenigvuldigen onrijpe bloedcellen zich en stapelen zich op in het beenmerg, waardoor de vorming van normale witte bloedcellen, rode bloedcellen en bloedplaatjes verdrongen wordt. Er ontstaat een tekort aan normale bloedcellen. Dit resulteert in bloedarmoede (door een tekort aan rode bloedcellen), bloedingen (door een tekort aan bloedplaatjes) en infecties (door een tekort aan witte bloedcellen).

AML komt voor bij zowel kinderen als volwassen. In Nederland worden per jaar ongeveer 20 kinderen gediagnosticeerd met AML; bij volwassenen ligt dit aantal hoger en neemt toe met de leeftijd (zie ook figuur 2 van hoofdstuk 1). Hoewel in de laatste decennia de "lange termijn overleving" van kinderen en volwassenen met AML sterk verbeterd is, genezen veel van deze patiënten niet. De behandeling is erop gericht om een complete remissie (CR) te bereiken, een situatie waarbij er geen AML meer worden waargenomen. Ondanks het feit dat meer dan de helft van de patiënten goed reageert op behandeling en CR bereikt, komt bij veel patiënten de ziekte na enige tijd weer terug. Vijf jaar na de diagnose van AML leeft nog ongeveer 60% van de kinderen. Voor volwassenen ligt dit percentage lager, met een 5-jaars-overleving van ongeveer 40% voor patiënten in de leeftijd van 18-60 jaar tot 10% 5-jaars-overleving voor oudere AML-patiënten. De behandeling bestaat op dit moment uit chemotherapie, eventueel gevolgd door een stamceltransplantatie. Om de overleving van patiënten met AML te verbeteren zijn nieuwe behandelstrategieën dus van groot belang.

Angiogenese en VEGF

De groei van tumoren is afhankelijk van de vorming van nieuwe bloedvaten uit al bestaande bloedvaten, een proces dat angiogenese wordt genoemd. Een goede bloedvoorziening zorgt namelijk voor de toevoer van zuurstof en voedingsstoffen, en de afvoer van afvalstoffen, allemaal nodig voor tumorcellen om zich te kunnen vermenigvuldigen. In het beenmerg van patiënten met AML is de vaatdichtheid ook hoger dan in het beenmerg van 'gezonde' mensen.
Tumorcellen zetten zelf het proces van angiogenese in gang door groeifactoren uit te scheiden. Een van de groeifactoren die een belangrijke rol speelt bij angiogenese is Vascular Endothelial Growth Factor (VEGF). AML patiënten met hogere VEGF waarden (in het plasma of in de AML cel) hebben een slechtere prognose. Bovendien is VEGF expressie in AML cellen gecorreleerd aan een verhoogde vaatdichtheid in het beenmerg. Deze aspecten in ogenschouw nemende lijken VEGF en angiogenese interessante aangrijpingspunten voor de ontwikkeling van nieuwe behandelingsstrategieën. In **hoofdstuk 2** presenteerden wij een overzicht van de tot het moment van schrijven bekende studies die betrekking hebben op VEGF en angiogenese in leukemie en andere vormen van 'bloedkanker' en 'lymfeklierkanker' (lymfomen), en beschreven de resultaten van geneesmiddelen die aangrijpen op VEGF en angiogenese (antiangiogene therapieën) in deze ziekten.

Antiangiogene therapie

VEGF wordt onder andere geproduceerd door AML cellen, en kan zich binden aan receptoren die op de buitenkant (membraan) van de cel zitten, de zogenoemde VEGF-receptoren. Tot nu toe zijn drie VEGF receptoren bekend, VEGFR1 t/m VEGFR3, waarvan VEGFR2 de belangrijkste is. Wanneer VEGF zich bindt aan één van de receptoren, verandert de receptor aan de binnenkant van de cel (dimerisatie en fosforylatie). Hierdoor worden er signalen doorgegeven binnenin de cel die verschillende processen in de cel aanzetten, waaronder de signaaltransductie routes PI3K/AKT en MAPK/ERK. Dit resulteert er uiteindelijk in dat de AML cel gaat delen en de leukemie in stand wordt gehouden en uitgroeit.
PTK787/ZK 222584 (PTK/ZK) is een geneesmiddel dat de fosforylatie van VEGF-receptoren blokkeert, waardoor de receptoren niet geactiveerd worden. In **hoofdstuk 3** hebben we gekeken naar het effect van PTK/ZK op AML cellen. We hebben vier verschillende AML cellijnen (verzameling van gelijke cellen die steeds weer opnieuw gekweekt kunnen worden) bestudeerd *in vitro* ('in glas', in kweeksystemen), en vonden dat PTK/ZK celdood induceerde in de drie AML cellijnen die VEGFR2 op de celmembraan hebben (HL-60, THP-1 en TF-1). Echter, de cellijn die geen VEGFR2 op de buitenkant van de cel had liet geen celdood zien als reactie op PTK/ZK (K562). Daarnaast liet PTK/ZK ook celdood zien in leukemie cellen van kinder-AML patiënten. Omdat chemotherapie tot op heden de standaard behandeling is voor AML, hebben we gekeken naar het additionele effect van PTK/ZK bovenop het effect van een chemotherapeuticum (Amsacrine). In zowel de AML cellijnen alsook de cellen van kinder-AML patiënten kon de dosis van het chemotherapeuticum verlaagd worden, en vervangen worden door het minder schadelijke PTK/ZK om uiteindelijk hetzelfde percentage dode AML cellen te verkrijgen.

Leukemische Stamcellen

Er wordt gedacht dat AML ontstaat uit een klein aantal cellen die in het beenmerg zitten, de Leukemische Stamcellen, ook wel Leukemie Initiërende Cellen genoemd (LICs). Het opnieuw krijgen van de ziekte AML (recidief genoemd) zou voortkomen uit hernieuwde groei van deze LICs die niet hebben gereageerd op de eerste behandeling. In **hoofdstuk 4** hebben we gekeken of VEGF/VEGFR signalering invloed heeft op de uitgroei van deze LICs. Met een '*in vitro* cocultuur systeem' waren we in staat om te laten zien dat kinder-AML LICs zichzelf kunnen vermeerderen tot een periode van minimaal 10 weken (in 9 van de 13 kinder-AML samples). In dit systeem werden de LICs gekweekt op MS5 stromale beenmergcellen (dit zijn steuncellen), om de 'natuurlijke' omgeving van de LICs in het beenmerg na te bootsen. In alle kinder-AML samples die we kweekten vonden we dat toevoeging van PTK/ZK resulteerde in verminderde uitgroei van de LICs vergeleken met de uitgroei zonder PTK/ZK. Er bleek geen relatie te bestaan tussen de expressie van de verschillende VEGF receptoren op de LICs en het effect dat PTK/ZK had op de uitgroei van de LICs. Wel vonden we dat de signaaltransductie route PI3K/Akt minder geactiveerd was wanneer de LICs met PTK/ZK werden gekweekt. Om te bestuderen of VEGF zelf ook effect heeft op de uitgroei van de LICs, kweekten we de LICs met toevoeging van VEGF of met Bevacizumab (een medicijn dat specifiek bindt aan VEGF, waardoor VEGF niet kan binden aan de receptoren, en deze niet geactiveerd kunnen worden). In beide situaties zagen we geen effect op de uitgroei van LICs, hetgeen suggereert dat VEGF alleen niet voldoende is om de groei van LICs te beïnvloeden. Concluderend liet deze studie zien dat PTK/ZK een effectieve benadering zou kunnen zijn om de LICs van kinderen met AML te elimineren.

Leukemie en stroma

Leukemiecellen bevinden zich in het beenmerg, en voor de ontwikkeling van leukemie lijkt de interactie met de omringende beenmergcellen een belangrijke rol te spelen. VEGF dat geproduceerd wordt door de AML-cellen stimuleert niet alleen de leukemiecellen zelf, maar beïnvloedt ook de micro-omgeving, zoals de stromale cellen ('steunweefsel' in het beenmerg, waaronder vetcellen en macrofagen) en endotheelcellen (cellen die de binnenkant van onder andere bloedvaten bedekken). Als reactie hierop scheiden deze cellen op hun beurt groeifactoren uit, die vervolgens weer kunnen zorgen voor groei van de leukemiecellen.
Om meer inzicht te krijgen in de interactie tussen AML cellen en de micro-omgeving, hebben we in **hoofdstuk 5** een AML cellijn getransduceerd met VEGF, wat inhoudt dat de AML cellen na transductie zo veranderd zijn dat ze veel VEGF produceren. Wanneer we deze VEGF-cellen *in vitro* kweekten, groeiden ze net zo snel als de controle-cellen (dezelfde AML cellijn, maar dan zonder VEGF transductie). Echter, na het injecteren van VEGF-cellen of controle-cellen in muizen *in vivo* ('in leven'), vormden de VEGF-cellen grotere tumoren, gepaard gaande met een toename van angiogenese in de tumoren. Verdere analyse van de VEGF-tumoren en de controle-tumoren suggereerde dat de Transforming Growth Factor ß (TGF-ß) signaaltransductie route minder actief is in de VEGF-tumoren. In de literatuur is beschreven dat een afname van de TGF-ß signaaltransductie route resulteert in een toename van celdeling, en dus een vermeerdering van cellen. Dit betekent dat de toegenomen groei van de VEGF-tumoren verklaard zou kunnen worden door minder activatie van TGF-ß signaaltransductie route. Logischerwijs zou dit het gevolg moeten zijn van de interactie met de micro-omgeving die aanwezig is *in vivo*; immers, *in vitro*

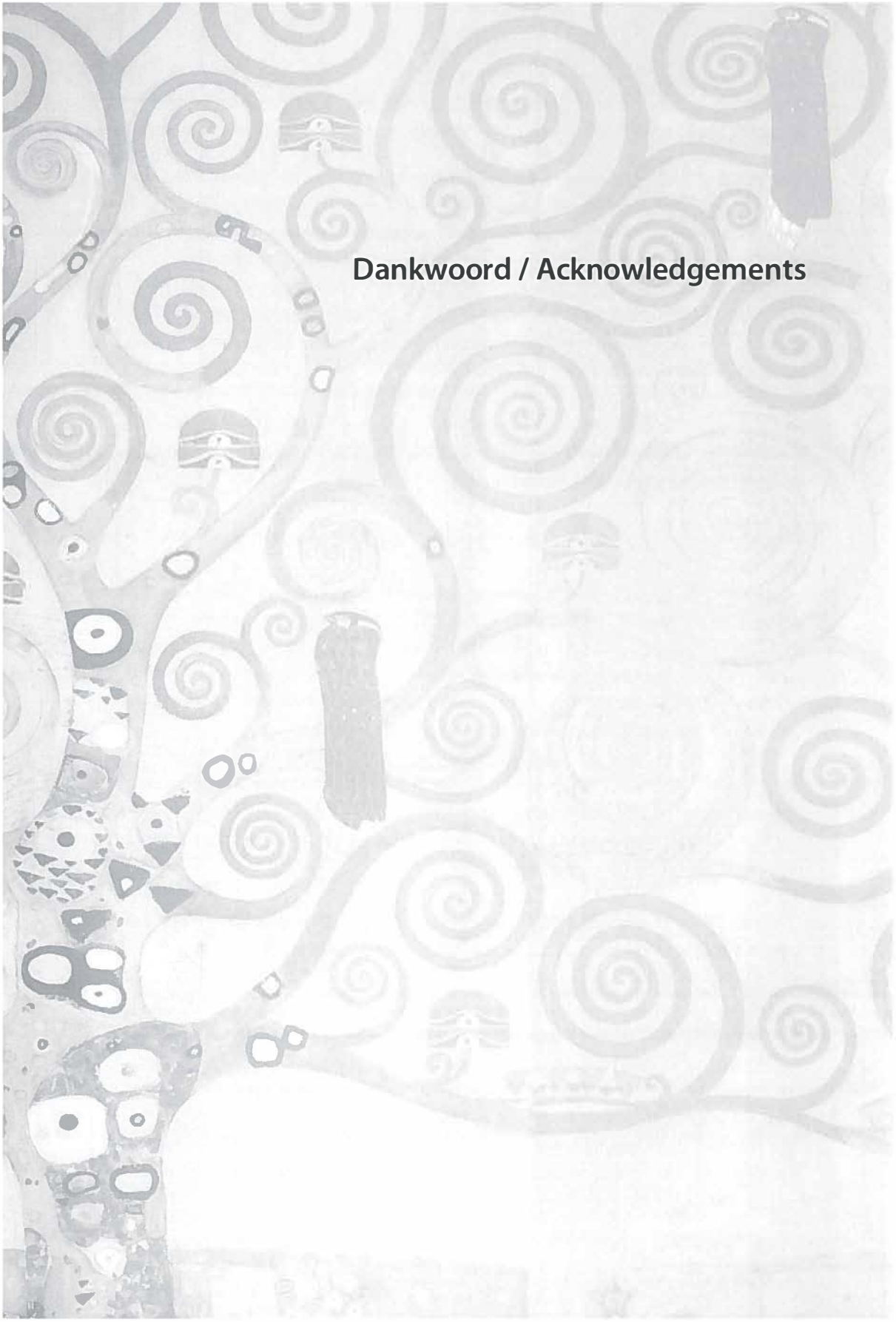
groeiden de VEGF-cellen en de controle-cellen even snel. Om dit verder te onderzoeken hebben we in de tumoren ook gekeken naar de groeifactoren die specifiek afkomstig waren van de muis, en vonden ook hier groeifactoren die betrokken zijn bij de TGF-ß signaaltransductie route. Deze studie suggereerde dus dat de interactie van de leukemiecellen met de micro-omgeving belangrijk is om ervoor te zorgen dat VEGF grotere tumoren produceert, via een afname van de TGF-ß signaaltransductie route.

Vaatmorfologie in het beenmerg van AML patiënten

Zoals hierboven beschreven wordt er in het beenmerg van AML patiënten meer angiogenese gevonden dat in beenmerg van 'gezonde' mensen. Echter, de morfologie (de vorm en structuur, letterlijk 'vormkunde') van het hoge aantal bloedvaten lijkt zeer uiteenlopend tussen de verschillende AML patiënten. In **hoofdstuk 6** bestudeerden we de morfologie van de bloedvaten in het beenmerg van AML patiënten bij diagnose. Wanneer we keken naar de endotheelcellen in het beenmerg (de cellen die de binnenkant van de bloedvaten vormen), konden we drie verschillende patronen onderscheiden: een groep met een 'low vessel count' (laag vaataantal, verschillende vaatgroottes), een groep genoemd 'angiogenic sprouting' (hoog vaataantal, dunne vaten), en een derde groep 'vessel hyperplasia' (hoog vaataantal, dikke vaten). Uit een muizenstudie is gebleken dat de hoeveelheid vrij VEGF gerelateerd is aan de morfologie van de vaten, waarbij veel vrij VEGF gerelateerd is aan een vaatpatroon met dikke vaten. In onze studie bleek dat VEGF uitgescheiden door de AML cellen hoger was in deze 'vessel hyperplasia' groep.

De aanwezigheid van verschillende vaatpatronen in beenmergbiopten roept de vraag op of het hebben van een bepaald vaatpatroon ook iets zegt over de prognose, of over de respons die de AML patiënt heeft op behandelingen met geneesmiddelen die VEGF/VEGFR signalering beïnvloeden. In **hoofdstuk 7** hebben we gebruik gemaakt van 93 beenmergbiopten van AML patiënten die meededen aan de HOVON81-studie, een studie die het effect onderzoekt van Bevacizumab (zoals hierboven beschreven, een medicijn dat specifiek bindt aan VEGF, waardoor VEGF niet kan binden aan de receptoren, en deze niet geactiveerd kunnen worden) als toevoeging op standaard chemotherapie. Patiënten met het vaatpatroon 'angiogenic sprouting' en 'low vessel count' hadden een significant kortere 'event-free survival (EFS)', de overlevingsperiode zonder dat de ziekte terugkomt. Ook de 'overall survival (OS)' (totale overleving) leek korter te zijn in de groep AML patiënten met 'angiogenic sprouting'. Vervolgens bestudeerden we of de vaatpatronen ook een voorspellende waarde zouden kunnen hebben. Naast twee bekende prognostische factoren, bleek ook het vaatpatroon een prognostische factor te zijn voor overleving. Tot slot wilden we nog weten of je met een bepaald vaatpatroon beter reageert op de behandeling met Bevacizumab. Interessant genoeg vonden we dat de patiënten met 'low vessel count' het meeste profijt hadden van de behandeling met Bevacizumab, hoewel genoemd moet worden dat de groepen voor deze analyse klein waren. Concluderend suggereerden deze resultaten dat het vaatpatroon van belang zou kunnen zijn om de AML overleving te voorspellen, en dat Bevacizumab wellicht een rol zou kunnen hebben in patiënten met een 'low vessel count'.

Tot slot werd een algemene samenvatting en discussie gegeven in **hoofdstuk 8**.



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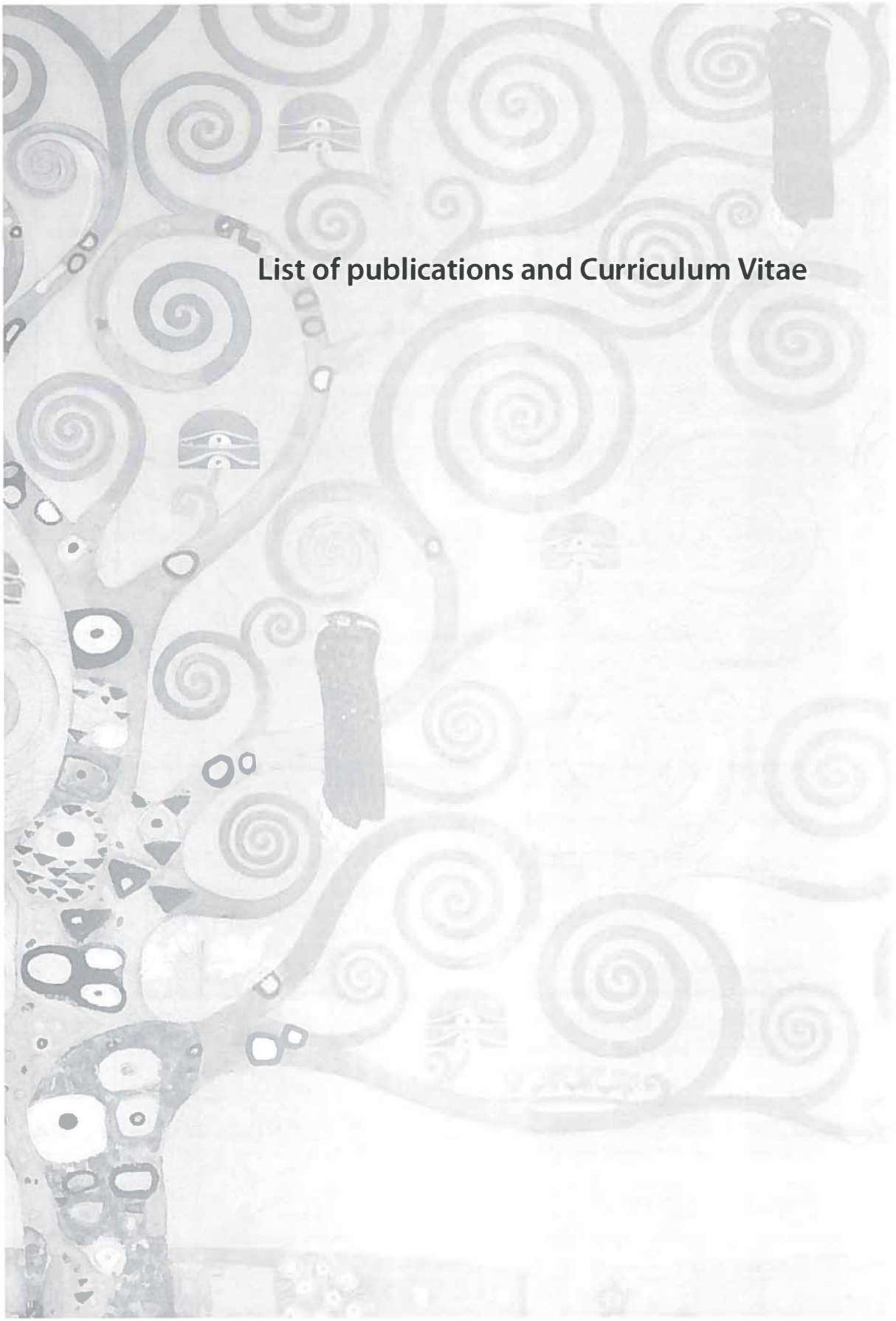
Alida



List of publications and Curriculum Vitae

List of publications

Weidenaar AC, ter Elst A, Kampen KR, Meeuwsen-de Boer T, de Jonge HJM, Scherpen FJG, den Dunnen WFA, Kamps WA, de Bont ESJM. Stromal interaction essential for Vascular Endothelial Growth Factor A induced tumor growth via Transforming Growth Factor ß signaling. British Journal of Cancer 2011 Dec 6;105(12):1856-63.

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Weidenaar AC, ter Elst A, van Montfort CAGM, Meeuwsen-de Boer T, Rosati S, Bargetzi M, Graux C, Gratwohl A, Lowenberg B, Vellenga E, Ossenkoppele GJ*, de Bont ESJM*. Patterns of Bone Marrow Micro Vessel Morphology in AML and High Risk MDS Predict Treatment Outcome Following Intensive Chemotherapy and Bevacizumab. Submitted. *shared last authorship.

Meeuwsen-de Boer T, Oosthoek J, **Weidenaar AC**, ter Elst A, van Montfort CAGM, Rosati S, Bargetzi M, Graux C, Gratwohl A, Lowenberg B, Vellenga E, Ossenkoppele GJ, de Bont ESJM. Nuclear HIF-1α is associated with different vascular patterns in bone marrow of AML patients. Work in progress.

Curriculum Vitae

Alida Cornelia Weidenaar werd geboren op 25 juni 1983 te Dokkum. Na het behalen van haar VWO-diploma aan het Dockingacollege te Dokkum in 2001, vertrok ze naar Groningen om Bewegingswetenschappen te studeren aan de Rijksuniversiteit Groningen. Een jaar later werd ze ingeloot voor de studie Geneeskunde. Als derdejaars student startte zij met het verrichten van wetenschappelijk onderzoek bij de afdeling Kinderoncologie van het Universitair Medisch Centrum Groningen (UMCG) onder begeleiding van dr. E.S.J.M. de Bont en prof. dr. W.A. Kamps. In 2006 begon ze aan een MD/PhD-traject onder hun begeleiding, hetgeen uiteindelijk resulteerde in dit proefschrift.

Tijdens haar studie was zij in het tweede en derde jaar als lid van het dagelijks bestuur actief betrokken bij de organisatie van het 'International Student Congress of Medical Sciences' (ISCOMS). Daarnaast hield zij zich bezig met de ontwikkelingen binnen het onderwijs van de faculteit door in haar vierde jaar zitting te nemen in de Onderzoek- en Onderwijsraad, en twee jaar een bestuursfunctie te bekleden binnen ProMed, het overkoepelend medezeggenschapsorgaan van geneeskundestudenten. Ook was zij als docent tutor drie jaar verbonden aan de faculteit der Medische Wetenschappen. Gedurende haar coschappen was zij betrokken bij de organisatie van het Landelijk Orgaan Coassistenten (LOCA) Congres.

In de klinische fase van haar studie liep ze met veel plezier haar eerste jaar coschappen in het UMCG en het Refaja Ziekenhuis Stadskanaal, en haar tweede jaar in het Martini Ziekenhuis Groningen. Van december 2009 tot maart 2010 liep ze een coschap in Wasso Hospitali te Wasso, Tanzania onder supervisie van tropenarts Christian van Rij. Zij sloot haar studie af met een keuzecoschap Urologie in het UMCG, en behaalde haar artsexamen in augustus 2010.

In 2011 werkte zij een jaar als ANIOS op de afdeling Urologie van het UMCG, onder begeleiding van prof. dr. J.M. Nijman en dr. I.J. de Jong. Vanaf januari 2012 is zij in het kader van haar vooropleiding werkzaam als arts-assistent op de afdeling Heelkunde van het Onze Lieve Vrouwe Gasthuis te Amsterdam, opleider dr. M.F. Gerhards. In 2014 zal haar opleiding tot uroloog worden voorgezet vanuit het UMCG.